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(54) Title: ANTIBODY DRUG CONJUGATES AND METHODS

(57) Abstract: The present invention relates to antibody-drug conjugate compounds of Formula I: Ab-(L-D)<sub>n</sub> where one or more  
maytansinoid drug moieties (D) are covalently linked by L to an antibody (Ab) which binds to an ErbB receptor, or which binds to  
one or more tumor-associated antigens or cell-surface receptors. These compounds may be used in methods of diagnosis or treatment  
of cancer, and other diseases and disorders.

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**ANTIBODY-DRUG CONJUGATES AND METHODS**

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This non-provisional application filed under 37 CFR §1.53(b), claims the benefit under 35 USC §119(e) of US Provisional Application Ser. No. 60/576, 517 filed on June 1, 2004 and US Provisional Application Ser. No. 60/616, 098 filed on October 5, 2004, each of which are incorporated by reference in their entirety.

**FIELD OF THE INVENTION**

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The invention relates generally to compounds with anti-cancer activity and more specifically to antibodies conjugated with chemotherapeutic maytansinoid drugs or toxins. The invention also relates to methods of using antibody-drug conjugate compounds for *in vitro*, *in situ*, and *in vivo* diagnosis or treatment of mammalian cells, or associated pathological conditions.

**BACKGROUND OF THE INVENTION**

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Antibody therapy has been established for the targeted treatment of patients with cancer, immunological and angiogenic disorders. The use of antibody-drug conjugates (ADC), i.e. immunoconjugates, for the local delivery of cytotoxic or cytostatic agents, i.e. drugs to kill or inhibit tumor cells in the treatment of cancer (Payne, G. (2003) Cancer Cell 3:207-212; Trail et al (2003) Cancer Immunol. Immunother. 52:328-337; Syrigos and Epenetos (1999) Anticancer Research 19:605-614; Niculescu-Duvaz and Springer (1997) Adv. Drug Del. Rev. 26:151-172; US 4975278) theoretically allows targeted delivery of the drug moiety to tumors, and intracellular accumulation therein, where systemic administration of these unconjugated drug agents may result in unacceptable levels of toxicity to normal cells as well as the tumor cells sought to be eliminated (Baldwin et al., (1986) Lancet pp. (Mar. 15, 1986):603-05; Thorpe, (1985) "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in Monoclonal Antibodies '84: Biological And Clinical Applications, A. Pinchera et al. (eds), pp. 475-506). Maximal efficacy with minimal toxicity is sought thereby. Efforts to design and refine ADC have focused on the selectivity of monoclonal antibodies (mAbs) as well as drug-linking and drug-releasing properties. Both polyclonal antibodies and monoclonal antibodies have been reported as useful in these strategies (Rowland et al., (1986) Cancer Immunol. Immunother. 21:183-87). Drugs used in these methods include daunomycin, doxorubicin, methotrexate, mitomycin, neocarzinostatin (Takahashi et al (1988) Cancer 61:881-888) and vindesine (Rowland et al., (1986) *supra*). Toxins used in antibody-toxin conjugates include bacterial toxins such as diphtheria toxin, plant toxins such as ricin (US 4753894; US 5629197; US 4958009; US 4956453), small molecule toxins such as geldanamycin (Mandler et al (2000) J. of the Nat. Cancer Inst. 92(19):1573-1581; Mandler et al (2000) Bioorganic & Med. Chem. Letters 10:1025-1028; Mandler et al (2002) Bioconjugate Chem. 13:786-791), maytansinoids (EP 1391213; Liu et al., (1996) Proc. Natl. Acad. Sci. USA 93:8618-8623), and calicheamicin (Lode et al (1998) Cancer Res. 58:2928; Hinman et al (1993) Cancer Res. 53:3336-3342). The toxins may effect their cytotoxic and cytostatic effects by mechanisms including

tubulin binding, DNA binding, or topoisomerase inhibition. Some cytotoxic drugs tend to be inactive or less active when conjugated to large antibodies or protein receptor ligands.

An antibody-radioisotope conjugate has been approved, ZEVALIN® (ibritumomab tiuxetan, Biogen/Idec) composed of a murine IgG1 kappa monoclonal antibody directed against CD20 antigen and <sup>111</sup>In or <sup>90</sup>Y radioisotope bound by a thiourea linker-chelator (Wiseman et al (2000) Eur. J. Nucl. Med. 27(7):766-77; Wiseman et al (2002) Blood 99(12):4336-42; Witzig et al (2002) J. Clin. Oncol. 20(10):2453-63; Witzig et al (2002) J. Clin. Oncol. 20(15):3262-69). MYLOTARG™ (gemtuzumab ozogamicin, Wyeth Pharmaceuticals), an antibody-drug conjugate composed of a hu CD33 antibody linked to calicheamicin, was approved in 2000 for the treatment of acute myeloid leukemia by injection (Drugs of the Future (2000) 25(7):686; US 4970198; US 5079233; US 5585089; US 5606040; US 5693762; US 5739116; US 5767285; US 5773001). Cantuzumab mertansine (Immunogen, Inc.), an antibody-drug conjugate composed of the huC242 antibody linked via the disulfide linker SPP to the maytansinoid drug moiety, DM1 (Xie et al (2004) J. of Pharm. and Exp. Ther. 308(3):1073-1082; Tolcher et al (2003) J. Clin. Oncology 21(2):211-222; US 5208020), underwent Phase I trials for the treatment of cancers that express CanAg, such as colon, pancreatic, gastric, and others. MLN-2704 (Millennium Pharm., BZL Biologics, Immunogen Inc.) is an antibody-drug conjugate composed of the anti-prostate specific membrane antigen (PSMA) monoclonal antibody linked to the maytansinoid drug moiety, DM1, under development for the potential treatment of prostate tumors. The same maytansinoid drug moiety, DM1, was linked through a non-disulfide linker, SMCC, to a mouse murine monoclonal antibody, TA.1 (Chari et al. (1992) Cancer Research 52:127-131) This conjugate was reported to be 200-fold less potent than the corresponding disulfide linker conjugate. The SMCC linker was considered therein to be "noncleavable" (also, see: US 4981979). HERCEPTIN® (trastuzumab) linked by SMCC to DM1 has been reported (WO 2005/037992).

In attempts to discover effective cellular targets for cancer diagnosis and therapy, researchers have sought to identify transmembrane or otherwise tumor-associated polypeptides that are specifically expressed on the surface of one or more particular type(s) of cancer cell as compared to on one or more normal non-cancerous cell(s). Often, such tumor-associated polypeptides are more abundantly expressed on the surface of the cancer cells as compared to on the surface of the non-cancerous cells. The identification of such tumor-associated cell surface antigen polypeptides, i.e. tumor-associated antigens (TAA), has given rise to the ability to specifically target cancer cells for destruction via antibody-based therapies.

Monoclonal antibody therapy has been established for the targeted treatment of patients with cancer, immunological and angiogenic disorders. An example of successful antibody therapy is HERCEPTIN® (trastuzumab), a recombinant DNA-derived humanized monoclonal antibody that selectively binds with high affinity in a cell-based assay ( $K_d = 5$  nM) to the extracellular domain of the human epidermal growth factor receptor2 protein, HER2 (ErbB2) (US 5821337; US 6054297; US 6407213; US 6639055; Coussens L, et al (1985) Science 230:1132-9; Slamon DJ, et al (1989) Science 244:707-12). Trastuzumab is an IgG1 kappa antibody that contains human framework regions with the complementarity-determining regions of a murine antibody (4D5) that binds to HER2. Trastuzumab binds to the HER2 antigen and thus inhibits the growth of cancerous cells. Because trastuzumab is a humanized antibody, it minimizes any HAMA response in patients. The humanized antibody against HER2 is produced by a mammalian cell (Chinese Hamster Ovary, CHO) suspension culture. The HER2 (or c-erbB2) proto-oncogene encodes a transmembrane receptor protein of

185kDa, which is structurally related to the epidermal growth factor receptor. HER2 protein overexpression is observed in 25%–30% of primary breast cancers and can be determined using an immunohistochemistry based assessment of fixed tumor blocks (Press MF, et al (1993) Cancer Res 53:4960-70. Trastuzumab has been shown, in both *in vitro* assays and in animals, to inhibit the proliferation of human tumor cells that overexpress

5 HER2 (Hudziak RM, et al (1989) Mol Cell Biol 9:1165-72; Lewis GD, et al (1993) Cancer Immunol Immunother; 37:255-63; Baselga J, et al (1998) Cancer Res. 58:2825-2831). Trastuzumab is a mediator of antibody-dependent cellular cytotoxicity, ADCC (Hotaling TE, et al (1996) [abstract]. Proc. Annual Meeting Am Assoc Cancer Res; 37:471; Pegram MD, et al (1997) [abstract]. Proc Am Assoc Cancer Res; 38:602; Sliwkowski et al (1999) Seminars in Oncology 26(4), Suppl 12:60-70; Yarden Y. and Sliwkowski, M. (2001)

10 Nature Reviews: Molecular Cell Biology, Macmillan Magazines, Ltd., Vol. 2:127-137). HERCEPTIN® is clinically active in patients with ErbB2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al, (1996) J. Clin. Oncol. 14:737-744). Although HERCEPTIN is a breakthrough in treating patients with ErbB2-overexpressing breast cancers that have received extensive prior anti-cancer therapy, the majority of the patients in this population fail to respond or respond only poorly to

15 HERCEPTIN treatment. Therefore, there is a significant clinical need for developing further HER2-directed cancer therapies for those patients with HER2-overexpressing tumors or other diseases associated with HER2 expression that do not respond, or respond poorly, to HERCEPTIN treatment. In addition to HER2, there is an opportunity to exploit other tumor-associated antigens with targeted therapies.

### SUMMARY

20 The present invention provides novel compounds with biological activity against cancer cells. The compounds may inhibit tumor growth in mammals and may be useful for treating human cancer patients.

The present invention relates to the delivery, transport, accumulation or retention of therapeutic antibody-drug conjugate (ADC) compounds inside cells. The invention is more particularly related to attaining high concentrations of active metabolite molecules in cancer cells. Intracellular targeting may be achieved by

25 methods and compounds which allow accumulation or retention of biologically active agents inside cells. Such effective targeting may be applicable to a variety of therapeutic formulations and procedures.

The surprising discovery has been made that antibody-drug conjugates with stable, non-disulfide linkers groups that attach a maytansinoid drug moiety to an antibody result in increased *in vitro* potency and *in vivo* efficacy. In addition, the antibody-drug conjugates show the unexpected result of better safety *in vivo*

30 relative to certain disulfide linker conjugates.

Antibody-drug conjugate (ADC) compounds comprise an antibody covalently attached by a linker to one or more maytansinoid drug moieties. ADC may be represented by Formula I:



where one or more maytansinoid drug moieties (D) are covalently linked by L to an antibody (Ab). Ab

35 is an antibody which binds to an ErbB receptor, or which binds to one or more tumor-associated antigens or cell-surface receptors. The linker L may be stable outside a cell, i.e. extracellular. The linker L, the maytansinoid drug moiety D, or the linker and the maytansinoid drug moiety taken together (L-D), do not comprise a disulfide group.

In one embodiment, a substantial amount of the drug moiety is not cleaved from the antibody until the antibody-drug conjugate enters a cell with a cell-surface receptor specific for the antibody of the antibody-drug conjugate, and the drug moiety is cleaved from the antibody when the antibody-drug conjugate does enter the cell.

5 In another embodiment, the ADC specifically binds to a receptor encoded by an ErbB gene, such as EGFR, HER2, HER3 and HER4. The ADC may specifically bind to the extracellular domain of the HER2 receptor. The ADC may inhibit growth of tumor cells which overexpress HER2 receptor.

10 In another embodiment, the antibody (Ab) of Formula I is a humanized antibody such as huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 or huMAb4D5-8 (trastuzumab).

Another aspect of the invention is a pharmaceutical composition including a Formula I compound, or a pharmaceutically acceptable salt or solvate thereof, and a pharmaceutically acceptable diluent, carrier, or excipient.

15 Another aspect provides a pharmaceutical combination comprising a Formula I compound and a second compound having anti-cancer properties or other therapeutic effects..

Another aspect includes diagnostic and therapeutic uses for the compounds and compositions disclosed herein.

20 Another aspect is a method for killing or inhibiting the proliferation of tumor cells or cancer cells comprising treating the cells with an amount of an antibody-drug conjugate, or a pharmaceutically acceptable salt or solvate thereof, being effective to kill or inhibit the proliferation of the tumor cells or cancer cells.

25 Another aspect are methods of treating cancer comprising administering to a patient a formulation of a Formula I compound. One method is for the treatment of cancer in a mammal, wherein the cancer is characterized by the overexpression of an ErbB receptor. The mammal optionally does not respond, or responds poorly, to treatment with an unconjugated anti-ErbB antibody. The method comprises administering to the mammal a therapeutically effective amount of an antibody-drug conjugate compound.

30 Another aspect is a method of inhibiting the growth of tumor cells that overexpress a growth factor receptor selected from the group consisting of HER2 receptor and EGF receptor comprising administering to a patient an antibody-drug conjugate compound which binds specifically to said growth factor receptor and a chemotherapeutic agent wherein said antibody-drug conjugate and said chemotherapeutic agent are each administered in amounts effective to inhibit growth of tumor cells in the patient.

Another aspect is a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor, comprising administering a combination of an antibody-drug conjugate compound of Formula I and a chemotherapeutic agent.

35 Another aspect is an assay method for detecting cancer cells comprising: exposing cells to an antibody-drug conjugate compound, and determining the extent of binding of the antibody-drug conjugate compound to the cells.

Another aspect concerns methods of screening ADC drug candidates for the treatment of a disease or disorder where the disease or disorder is characterized by the overexpression of HER2.

40 Another aspect includes articles of manufacture, i.e. kits, comprising an antibody-drug conjugate, a container, and a package insert or label indicating a treatment.

Another aspect includes methods of treating a disease or disorder characterized by the overexpression of HER2 in a patient with the antibody-drug conjugate compounds.

Another aspect includes methods of making, methods of preparing, methods of synthesis, methods of conjugation, and methods of purification of the antibody-drug conjugate compounds, and the intermediates for the preparation, synthesis, and conjugation of the antibody-drug conjugate compounds.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an *in vitro*, cell proliferation assay with SK-BR-3 cells treated with antibody-drug conjugates:

—□— trastuzumab-SPP-DM1, —Δ— trastuzumab-SPDP-DM1, and —○— trastuzumab-SMCC-DM1.

Figure 2 shows an *in vitro*, cell proliferation assay with BT-474 cells treated with antibody-drug conjugates:

—□— trastuzumab-SPP-DM1, —Δ— trastuzumab-SPDP-DM1, and —○— trastuzumab-SMCC-DM1.

Figure 3 shows an *in vitro*, cell proliferation assay with MCF7 cells treated with antibody-drug conjugates: —□— trastuzumab-SPP-DM1, —Δ— trastuzumab-SPDP-DM1, and —○— trastuzumab-SMCC-DM1.

Figure 4 shows an *in vitro*, cell proliferation assay with MDA-MB-468 cells treated with antibody-drug conjugates: —□— trastuzumab-SPP-DM1, —Δ— trastuzumab-SPDP-DM1, and —○— trastuzumab-SMCC-DM1.

Figure 5 shows the serum clearance in beige nude mice without tumors of trastuzumab-SMCC-DM1 vs. trastuzumab-SPP-DM1, measuring conjugate and total antibody serum concentration at six time points (5 minutes, 1 hour, 6 hours, 24 hours, 72, 168 hours post-dose) over 7 days.

Figure 6 shows the stability over time in nude mice without tumors of the conjugates: trastuzumab-SPDP-DM1, trastuzumab-SPP-DM1, trastuzumab-SPP-DM3, trastuzumab-SPP-DM4, and trastuzumab-SMCC-DM1, measuring serum concentration at six time points (5 minutes, 1 hour, 6 hours, 24 hours, 72, 168 hours post-dose) over 7 days.

Figure 7 shows the measurement of serum concentrations of total trastuzumab / trastuzumab-SMCC-DM1, and total trastuzumab / trastuzumab-SPP-DM1 in mice, 7 days after treatment, with and without tumor.

Figure 8 shows a plasma concentration clearance study after administration of 10 mg/kg of trastuzumab-SPP-DM1 to 4 subject rats. Concentrations of total antibody and trastuzumab-SPP-DM1 were measured. (tr = trastuzumab)

Figure 9 shows a plasma concentration clearance study after administration of 10 mg/kg of trastuzumab-SMCC-DM1 to 4 subject rats. Concentrations of total antibody and trastuzumab-SMCC-DM1 were measured.

Figure 10 shows the mean tumor volume change over time in mice dosed with: Vehicle (PBS pH 6.5), trastuzumab-SPP-DM1 (370  $\mu\text{g DM1/m}^2$ ), and trastuzumab-SMCC-DM1 (330  $\mu\text{g DM1/m}^2$ ) where dose refers to the dose of DM1 administered.

Figure 11 shows the mean tumor volume change over time in athymic nude mice with Fo5 tumor allografts dosed on Day 0 with: Vehicle (PBS pH 6.5), 10 mg/kg trastuzumab-SIAB-DM1 (3.4 DM1/Ab; 168  $\mu\text{g DM1/kg}$ ), and 10 mg/kg trastuzumab-SMCC-DM1 (3.2 DM1/Ab; 158  $\mu\text{g DM1/kg}$ ) where dose refers to the dose amount of the antibody-drug conjugate administered.

Figure 12 shows the mean tumor volume change over time in MMTV-Her2 Fo 5 beige nude mice (seven each group, all with tumors, Ti = 7) by single injection with Vehicle (PBS pH 6.5), 10 mg/kg trastuzumab-

SPP-DM1, 10 mg/kg trastuzumab-SPP-DM4, 10 mg/kg trastuzumab-SPP-DM3, and 10 mg/kg trastuzumab-SMCC-DM1.

Figure 13 shows the time to double tumor volume and log cell kill analysis for Vehicle (PBS pH 6.5), trastuzumab-SPP-DM1, trastuzumab-SPP-DM4, trastuzumab-SPP-DM3, and trastuzumab-SMCC-DM1 in HER2-Fo5 tumors.

Figure 14 shows the change in body weight over time of rats dosed with: Vehicle (10 mM sodium succinate, 100 mg/mL sucrose, 0.1% Tween 20, pH 5.0), trastuzumab-SPP-DM1 (1860  $\mu\text{g DM1/m}^2$ ), trastuzumab-SMCC-DM1 (1860  $\mu\text{g DM1/m}^2$ ), trastuzumab-SMCC-DM1 (3260  $\mu\text{g DM1/m}^2$ ), and free DM1 (650  $\mu\text{g/m}^2$ ).

Figure 15 shows a liver function test measured in AST units per liter over time in the rat model dosed with: Vehicle (10 mM sodium succinate, 100 mg/mL sucrose, 0.1% Tween 20, pH 5.0), trastuzumab-SPP-DM1 (22.3 mg/kg), trastuzumab-SMCC-DM1 (10 mg/kg), trastuzumab-SMCC-DM1 (25 mg/kg), and free DM1.

Figure 16 shows a safety profile measured in PLT units in cells per liter over time in the rat model dosed with: Vehicle (10 mM sodium succinate, 100 mg/mL sucrose, 0.1% Tween 20, pH 5.0), trastuzumab-SPP-DM1 (22.3 mg/kg), trastuzumab-SMCC-DM1 (10 mg/kg), trastuzumab-SMCC-DM1 (25 mg/kg), and free DM1.

Figure 17 shows an in vitro, cell proliferation assay with HT1080EphB2 (C8) cells treated with antibody-drug conjugates:  $\blacktriangle$  - antiEphB2R 2H9-SPP-DM1, and  $\blacktriangledown$  - antiEphB2R 2H9-SMCC-DM1.

## DETAILED DESCRIPTION OF EXEMPLARY EMBODIMENTS

Reference will now be made in detail to certain embodiments of the invention, examples of which are illustrated in the accompanying structures and formulas. While the invention will be described in conjunction with the enumerated embodiments, it will be understood that they are not intended to limit the invention to those embodiments. On the contrary, the invention is intended to cover all alternatives, modifications, and equivalents, which may be included within the scope of the present invention as defined by the claims.

One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. The present invention is in no way limited to the methods and materials described.

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs, and are consistent with: Singleton *et al.*, (1994) *Dictionary of Microbiology and Molecular Biology*, 2nd Ed., J. Wiley & Sons, New York, NY; and Janeway, C., Travers, P., Walport, M., Shlomchik (2001) *Immuno Biology*, 5th Ed., Garland Publishing, New York.

## DEFINITIONS

Unless stated otherwise, the following terms and phrases as used herein are intended to have the following meanings:

When trade names are used herein, applicants intend to independently include the trade name product formulation, the generic drug, and the active pharmaceutical ingredient(s) of the trade name product.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, dimers, multimers, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments, so long as they exhibit the desired biological activity (Miller et al (2003) *Jour. of Immunology* 170:4854-4861). Antibodies may be murine, human, humanized, chimeric, or derived from other species. An antibody is a protein generated by the immune system that is capable of recognizing and binding to a specific antigen. (Janeway, C., Travers, P., Walport, M., Shlomchik (2001) *Immuno Biology, 5th Ed.*, Garland Publishing, New York). A target antigen generally has numerous binding sites, also called epitopes, recognized by CDRs on multiple antibodies. Each antibody that specifically binds to a different epitope has a different structure. Thus, one antigen may have more than one corresponding antibody. An antibody includes a full-length immunoglobulin molecule or an immunologically active portion of a full-length immunoglobulin molecule, i.e., a molecule that contains an antigen binding site that immunospecifically binds an antigen of a target of interest or part thereof, such targets including but not limited to, cancer cell or cells that produce autoimmune antibodies associated with an autoimmune disease. The immunoglobulin disclosed herein can be of any type (e.g., IgG, IgE, IgM, IgD, and IgA), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The immunoglobulins can be derived from any species. In one aspect, however, the immunoglobulin is of human, murine, or rabbit origin.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab')<sub>2</sub>, and Fv fragments; diabodies; linear antibodies; fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, CDR (complementary determining region), and epitope-binding fragments of any of the above which immunospecifically bind to cancer cell antigens, viral antigens or microbial antigens, single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al (1975) *Nature* 256:495, or may be made by recombinant DNA methods (see, US 4816567). The monoclonal antibodies may also be isolated from phage antibody libraries using the techniques described in Clackson et al (1991) *Nature*, 352:624-628; Marks et al (1991) *J. Mol. Biol.*, 222:581-597; for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or

belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (US 4816567; and Morrison et al (1984) Proc. Natl. Acad. Sci. USA, 81:6851-6855). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g., Old World Monkey or Ape) and human constant region sequences.

An "intact antibody" herein is one comprising a VL and VH domains, as well as a light chain constant domain (CL) and heavy chain constant domains, CH1, CH2 and CH3. The constant domains may be native sequence constant domains (e.g., human native sequence constant domains) or amino acid sequence variant thereof. The intact antibody may have one or more "effector functions" which refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody. Examples of antibody effector functions include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; and down regulation of cell surface receptors such as B cell receptor and BCR.

Depending on the amino acid sequence of the constant domain of their heavy chains, intact antibodies can be assigned to different "classes." There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into "subclasses" (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called  $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$ , and  $\mu$ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

An "ErbB receptor" is a receptor protein tyrosine kinase which belongs to the ErbB receptor family which are important mediators of cell growth, differentiation and survival. The ErbB receptor family includes four distinct members including epidermal growth factor receptor (EGFR, ErbB1, HER1), HER2 (ErbB2 or p185<sup>neu</sup>), HER3 (ErbB3) and HER4 (ErbB4 or tyro2). A panel of anti-ErbB2 antibodies has been characterized using the human breast tumor cell line SKBR3 (Hudziak et al (1989) Mol. Cell. Biol. 9(3):1165-1172.

Maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel reduced cellular proliferation to a lesser extent in this assay. The antibody 4D5 was further found to sensitize ErbB2-overexpressing breast tumor cell lines to the cytotoxic effects of TNF- $\alpha$  (US 5677171). The anti-ErbB2 antibodies discussed in Hudziak et al are further characterized in Fendly et al (1990) Cancer Research 50:1550-1558; Kotts et al. (1990) In Vitro 26(3):59A; Sarup et al. (1991) Growth Regulation 1:72-82; Shepard et al. J. (1991) Clin. Immunol. 11(3):117-127; Kumar et al. (1991) Mol. Cell. Biol. 11(2):979-986; Lewis et al. (1993) Cancer Immunol. Immunother. 37:255-263; Pietras et al. (1994) Oncogene 9:1829-1838; Vitetta et al. (1994) Cancer Research 54:5301-5309; Sliwkowski et al. (1994) J. Biol. Chem. 269(20):14661-14665; Scott et al. (1991) J. Biol. Chem. 266:14300-5; D'souza et al. Proc. Natl. Acad. Sci. (1994) 91:7202-7206; Lewis et al. (1996) Cancer Research 56:1457-1465; and Schaefer et al. (1997) Oncogene 15:1385-1394.

Other anti-ErbB2 antibodies with various properties have been described in Franklin et al (2004) Cancer Cell 5:317-328; Tagliabue et al (1991) Int. J. Cancer 47:933-937; McKenzie et al (1989) Oncogene 4:543-548; Maier et al (1991) Cancer Res. 51:5361-5369; Bacus et al (1990) Molecular Carcinogenesis 3:350-362; Stancovski et al (1991) PNAS (USA) 88:8691-8695; Bacus et al (1992) Cancer Research 52:2580-2589; Xu et al (1993) Int. J. Cancer 53:401-408; WO94/00136; Kasprzyk et al (1992) Cancer Research 52:2771-2776;

Hancock et al (1991) Cancer Res. 51:4575-4580; Shawver et al (1994) Cancer Res. 54:1367-1373; Arteaga et al (1994) Cancer Res. 54:3758-3765; Harwerth et al (1992) J. Biol. Chem. 267:15160-15167; US 5783186; and Klapper et al (1997) Oncogene 14:2099-2109.

Sequence identity screening has resulted in the identification of two other ErbB receptor family members; ErbB3 (US 5,183,884; US 5,480,968; Kraus et al (1989) PNAS (USA) 86:9193-9197) and ErbB4 (EP 599274; Plowman et al (1993) Proc. Natl. Acad. Sci. USA, 90:1746-1750; and Plowman et al (1993) Nature 366:473-475). Both of these receptors display increased expression on at least some breast cancer cell lines.

The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a "native sequence" ErbB receptor or an "amino acid sequence variant" thereof. The ErbB receptor may be native sequence human ErbB receptor. Accordingly, a "member of the ErbB receptor family" is EGFR (ErbB1), ErbB2, ErbB3, ErbB4 or any other ErbB receptor currently known or to be identified in the future.

The terms "ErbB1", "epidermal growth factor receptor", "EGFR" and "HER1" are used interchangeably herein and refer to EGFR as disclosed, for example, in Carpenter et al (1987) Ann. Rev. Biochem. 56:881-914, including naturally occurring mutant forms thereof (e.g., a deletion mutant EGFR as in Humphrey et al., (1990) PNAS (USA), 87:4207-4211). The term erbB1 refers to the gene encoding the EGFR protein product. Antibodies against HER1 are described, for example, in Murthy et al (1987) Arch. Biochem. Biophys., 252:549-560 and in WO 95/25167.

The term "ERRP", "EGF-Receptor Related Protein", "EGFR Related Protein" and "epidermal growth factor receptor related protein" are used interchangeably herein and refer to ERRP as disclosed, for example in US 6399743 and US 2003/0096373.

The expressions "ErbB2" and "HER2" are used interchangeably herein and refer to human HER2 protein described, for example, in Semba et al (1985) PNAS (USA), 82:6497-6501 and Yamamoto et al (1986) Nature, 319:230-234 (Genbank accession number X03363). The term "erbB2" refers to the gene encoding human ErbB2 and "neu" refers to the gene encoding rat p185neu.

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in US 5183884; US 5480968; Kraus et al (1989) PNAS (USA) 86:9193-9197. Antibodies against ErbB3 are known in the art (US 5183884; US 5480968; WO 97/35885).

The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in EP Pat Appln No 599,274; Plowman et al., Proc. Natl. Acad. Sci. USA, 90:1746-1750 (1993); and Plowman et al., Nature, 366:473-475 (1993), including isoforms thereof, e.g., as disclosed in WO 99/19488. Antibodies against HER4 are described, for example, in WO 02/18444.

Antibodies to ErbB receptors are available commercially from a number of sources, including, for example, Santa Cruz Biotechnology, Inc., California, USA.

By "ErbB ligand" is meant a polypeptide which binds to and/or activates an ErbB receptor. The ErbB ligand may be a native sequence human ErbB ligand such as epidermal growth factor (EGF) (Savage et al (1972) J. Biol. Chem., 247:7612-7621); transforming growth factor alpha (TGF- $\alpha$ ) (Marquardt et al (1984) Science 223:1079-1082); amphiregulin also known as schwannoma or keratinocyte autocrine growth factor (Shoyab et al (1989) Science 243:1074-1076; Kimura et al (1990) Nature 348:257-260; and Cook et al (1991)

Mol. Cell. Biol., 11:2547-2557); betacellulin (Shing et al (1993) Science 259:1604-1607; and Sasada et al (1993) Biochem. Biophys. Res. Commun. 190:1173); heparin-binding epidermal growth factor (HB-EGF) (Higashiyama et al (1991) Science 251:936-939); epiregulin (Toyoda et al (1995) J. Biol. Chem. 270:7495-7500; and Komurasaki et al (1997) Oncogene 15:2841-2848); a heregulin (see below); neuregulin-2 (NRG-2) (Carraway et al., Nature, 387:512-516 (1997)); neuregulin-3 (NRG-3) (Zhang et al (1997) Proc. Natl. Acad. Sci., 94:9562-9567); neuregulin-4 (NRG-4) (Harari et al (1999) Oncogene, 18:2681-89) or cripto (CR-1) (Kannan et al (1997) J. Biol. Chem., 272(6):3330-3335). ErbB ligands which bind EGFR include EGF; TGF- $\alpha$ , amphiregulin, betacellulin, HB-EGF and epiregulin. ErbB ligands which bind ErbB3 include heregulins. ErbB ligands capable of binding ErbB4 include betacellulin, epiregulin, HB-EGF, NRG-2, NRG-3, NRG-4 and heregulins. The ErbB ligand may also be a synthetic ErbB ligand. The synthetic ligand may be specific for a particular ErbB receptor, or may recognize particular ErbB receptor complexes. An example of a synthetic ligand is the synthetic heregulin/EGF chimera biregulin (see, for example, Jones et al (1999) FEBS Letters, 447:227-231, which is incorporated by reference).

"Heregulin" (HRG) refers to a polypeptide encoded by the heregulin gene product as disclosed in US 5641869 or Marchionni et al (1993) Nature 362:312-318. Examples of heregulins include heregulin- $\alpha$ , heregulin- $\beta$ 1, heregulin- $\beta$ 2 and heregulin- $\beta$ 3 (Holmes et al (1992) Science 256:1205-1210; and US 5641869); neu differentiation factor (NDF) (Peles et al (1992) Cell 69: 205-216); acetylcholine receptor-inducing activity (ARIA) (Falls et al (1993) Cell 72:801-815); glial growth factors (GGFs) (Marchionni et al (1993) Nature, 362:312-318); sensory and motor neuron derived factor (SMDF) (Ho et al (1995) J. Biol. Chem. 270:14523-14532);  $\gamma$ -heregulin (Schaefer et al (1997) Oncogene, 15:1385-1394). The term includes biologically active fragments and/or amino acid sequence variants of a native sequence HRG polypeptide, such as an EGF-like domain fragment thereof (e.g., HRG $\beta$ 1177-244 ).

"ErbB hetero-oligomer" is a noncovalently associated oligomer comprising at least two different ErbB receptors. An "ErbB dimer" is a noncovalently associated oligomer that comprises two different ErbB receptors. Such complexes may form when a cell expressing two or more ErbB receptors is exposed to an ErbB ligand. ErbB oligomers, such as ErbB dimers, can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al (1994) J. Biol. Chem., 269(20):14661-14665, for example. Examples of such ErbB hetero-oligomers include EGFR-ErbB2 (also referred to as HER1/HER2), ErbB2-ErbB3 (HER2/HER3) and ErbB3-ErbB4 (HER3/HER4) complexes. Moreover, the ErbB hetero-oligomer may comprise two or more ErbB2 receptors combined with a different ErbB receptor, such as ErbB3, ErbB4 or EGFR (ErbB1). Other proteins, such as a cytokine receptor subunit (e.g., gp130) may be included in the hetero-oligomer.

By "ligand activation of an ErbB receptor" is meant signal transduction (e.g., that caused by an intracellular kinase domain of an ErbB receptor phosphorylating tyrosine residues in the ErbB receptor or a substrate polypeptide) mediated by ErbB ligand binding to a ErbB hetero-oligomer comprising the ErbB receptor of interest. Generally, this will involve binding of an ErbB ligand to an ErbB hetero-oligomer which activates a kinase domain of one or more of the ErbB receptors in the hetero-oligomer and thereby results in phosphorylation of tyrosine residues in one or more of the ErbB receptors and/or phosphorylation of tyrosine residues in additional substrate polypeptides(s). ErbB receptor activation can be quantified using various tyrosine phosphorylation assays.

A "native sequence" polypeptide is one which has the same amino acid sequence as a polypeptide (e.g., ErbB receptor or ErbB ligand) derived from nature. Such native sequence polypeptides can be isolated from nature or can be produced by recombinant or synthetic means. Thus, a native sequence polypeptide can have the amino acid sequence of naturally occurring human polypeptide, murine polypeptide, or polypeptide from any other mammalian species.

The term "amino acid sequence variant" refers to polypeptides having amino acid sequences that differ to some extent from a native sequence polypeptide. Ordinarily, amino acid sequence variants will possess at least about 70% sequence identity with at least one receptor binding domain of a native ErbB ligand or with at least one ligand binding domain of a native ErbB receptor, or at least about 80%, or at least about 90% homologous with such receptor or ligand binding domains. The amino acid sequence variants possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence of the native amino acid sequence.

"Sequence identity" is defined as the percentage of residues in the amino acid sequence variant that are identical after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Methods and computer programs for the alignment are well known in the art. One such computer program is "Align 2," authored by Genentech, Inc., which was filed with user documentation in the United States Copyright Office, Washington, DC 20559, on December 10, 1991.

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (e.g., Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, (1991) Annu. Rev. Immunol, 9:457-92. To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay may be performed (US 5500362; US 5821337). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, e.g., in a animal model such as that disclosed in Clynes et al (1998) PNAS (USA), 95:652-656.

"Maytansinoid drug moiety" means the substructure of an antibody-drug conjugate that has the structure of a maytansine compound. Maytansine was first isolated from the east African shrub *Maytenus serrata* (US 3896111). Subsequently, it was discovered that certain microbes also produce maytansinoids, such as maytansinol and C-3 maytansinol esters (US 4151042). Synthetic maytansinol and maytansinol analogues have been reported. See US Patent Nos. 4137230; 4248870; 4256746; 4260608; 4265814; 4294757; 4307016; 4308268; 4308269; 4309428; 4313946; 4315929; 4317821; 4322348; 4331598; 4361650; 4364866; 4424219; 4450254; 4362663; and 4371533, and Kawai et al (1984) Chem. Pharm. Bull. 3441-3451), each of which are expressly incorporated by reference.

The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody, such as a native sequence human FcR. FcR may bind an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains

thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (See review M. in Daëron, *Annu. Rev. Immunol.*, 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol.*, 9:457-92 (1991); Capel et al (1994) *Immunomethods*, 4:25-34; and de Haas et al (1995) *J. Lab. Clin. Med.*, 126:330-41. Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al (1976) *J. Immunol.* 117:587, and Kim et al (1994) *J. Immunol.*, 24:249).

"Complement dependent cytotoxicity" or "CDC" refers to the ability of a molecule to lyse a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (*e.g.*, an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, *e.g.*, as described in Gazzano-Santoro et al (1996) *J. Immunol. Methods*, 202:163, may be performed.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains. Each light chain has a variable domain at one end (VL) and a constant domain at its other end. The constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al (1991) *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region generally comprises amino acid residues from a "complementarity determining region" or "CDR" (*e.g.*, residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al *supra*) and/or those residues from a "hypervariable loop" (*e.g.*, residues 26-32 (L1), 50-52

(L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk (1987) J. Mol. Biol., 196:901-917). "Framework Region" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')<sub>2</sub> fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear at least one free thiol group. F(ab')<sub>2</sub> antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called kappa ( $\kappa$ ) and lambda ( $\lambda$ ), based on the amino acid sequences of their constant domains.

"Single-chain Fv" or "scFv" mean single chain variable region antibody fragments which comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. The Fv polypeptide may further comprise a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding (Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994). Anti-ErbB2 antibody scFv fragments are described in WO 93/16185; US 5571894; US 5587458.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a variable heavy domain (VH) connected to a variable light domain (VL) in the same polypeptide chain (VH - VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al (1993) Proc. Natl. Acad. Sci. USA 90:6444-6448.

"Humanized" forms of non-human (e.g., rodent) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues.

Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al (1986) *Nature*, 321:522-525; Riechmann et al (1988) *Nature* 332:323-329; and Presta, (1992) *Curr. Op. Struct. Biol.*, 2:593-596.

Humanized anti-ErbB2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 (HERCEPTIN®, trastuzumab) as described in Table 3 of US 5821337 expressly incorporated herein by reference; humanized 520C9 (WO 93/21319) and humanized 2C4 antibodies.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. The antibody may be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, or more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup protein sequencer, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

An antibody "which binds" an antigen of interest, *e.g.*, ErbB2 antigen, is one capable of binding that antigen with sufficient affinity such that the antibody is useful in targeting a cell expressing the antigen. Where the antibody is one which binds ErbB2, it will usually preferentially bind ErbB2 as opposed to other ErbB receptors, and may be one which does not significantly cross-react with other proteins such as EGFR, ErbB3 or ErbB4. In such embodiments, the extent of binding of the antibody to these non-ErbB2 proteins (*e.g.*, cell surface binding to endogenous receptor) will be less than 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA). Sometimes, the anti-ErbB2 antibody will not significantly cross-react with the rat neu protein, *e.g.*, as described in Schecter et al (1984) *Nature* 312:513, and Drebin et al (1984) *Nature*, 312:545-548.

An antibody which "blocks" ligand activation of an ErbB receptor reduces or prevents such activation, wherein the antibody is able to block ligand activation of the ErbB receptor substantially more effectively than monoclonal antibody 4D5, *e.g.*, about as effectively as monoclonal antibodies 7F3 or 2C4 or Fab fragments thereof. For example, the antibody that blocks ligand activation of an ErbB receptor may be one which is about 50-100% more effective than 4D5 at blocking formation of an ErbB hetero-oligomer. Blocking of ligand activation of an ErbB receptor can occur by any means, *e.g.*, by interfering with: ligand binding to an ErbB receptor, ErbB complex formation, tyrosine kinase activity of an ErbB receptor in an ErbB complex and/or phosphorylation of tyrosine kinase residue(s) in or by an ErbB receptor.

An antibody having a "biological characteristic" of a designated antibody, such as the monoclonal antibody designated 2C4 (Omnitarg, Genentech, Inc.), is one which possesses one or more of the biological characteristics of that antibody which distinguish it from other antibodies that bind to the same antigen (*e.g.*, ErbB2). For example, an antibody with a biological characteristic of 2C4 may block HRG activation of an ErbB hetero-oligomer comprising ErbB2 and ErbB3, ErbB1 or ErbB4; block EGF, TGF- $\alpha$ , HB-EGF, epiregulin and/or amphiregulin activation of an ErbB receptor comprising EGFR and ErbB2; block EGF, TGF- $\alpha$  and/or HRG mediated activation of MAPK; and/or bind the same epitope in the extracellular domain of ErbB2 as that bound by 2C4 (*e.g.*, which blocks binding of monoclonal antibody 2C4 to ErbB2).

Unless indicated otherwise, the expression "monoclonal antibody 2C4" refers to an antibody that has antigen binding residues of, or derived from, the murine 2C4 antibody of the Examples below. For example, the monoclonal antibody 2C4 may be murine monoclonal antibody 2C4 or a variant thereof, such as humanized antibody 2C4, possessing antigen binding amino acid residues of murine monoclonal antibody 2C4 (WO 01/00245). Unless indicated otherwise, the expression "rhuMAb 2C4" when used herein refers to an antibody comprising the variable light (VL) and variable heavy (VH) sequences of SEQ ID Nos. 3 and 4, respectively, fused to human light and heavy IgG1 (non-A allotype) constant region sequences optionally expressed by a Chinese Hamster Ovary (CHO) cell (WO 01/00245).

Unless indicated otherwise, the term "monoclonal antibody 4D5" refers to an antibody that has antigen binding residues of, or derived from, the murine 4D5 antibody (ATCC CRL 10463). For example, the monoclonal antibody 4D5 may be murine monoclonal antibody 4D5 or a variant thereof, such as a humanized 4D5, possessing antigen binding residues of murine monoclonal antibody 4D5. Exemplary humanized 4D5 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 (HERCEPTIN®) as in US 5821337.

A "growth inhibitory agent" refers to a compound or composition which inhibits growth of a cell, *e.g.* an ErbB expressing cancer cell either *in vitro* or *in vivo*. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of ErbB expressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest (The Molecular Basis of Cancer, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13). Examples of "growth inhibitory" antibodies are those which bind to ErbB2 and inhibit the growth of cancer cells overexpressing ErbB2. Growth inhibitory anti-ErbB2 antibodies may inhibit growth of SK-BR-3 breast tumor cells in cell culture by greater than 20%, or greater than 50% (*e.g.*, from about 50% to about 100%) at an antibody concentration of about 0.5 to 30  $\mu$ g/ml, where the growth inhibition is determined six days after exposure of the SK-BR-3 cells to the antibody (US 5677171).

An antibody which "induces cell death" is one which causes a viable cell to become nonviable. The cell is generally one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. The cell may be a cancer cell, *e.g.*, a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. *In vitro*, the cell may be a SK-BR-3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death *in vitro* may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody-dependent cell-mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using

heat inactivated serum (*i.e.*, in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al (1995) Cytotechnology, 17:1-11) or 7AAD can be assessed relative to untreated cells. Cell death-inducing antibodies are those which induce PI uptake in the PI uptake assay in BT474 cells (see below).

An antibody which "induces apoptosis" is one which induces programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is usually one which overexpresses the ErbB2 receptor, including a tumor cell, *e.g.*, a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SK-BR-3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell.

The terms "treat" or "treatment" refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change or disorder, such as the development or spread of cancer. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already with the condition or disorder as well as those prone to have the condition or disorder or those in which the condition or disorder is to be prevented.

A "disorder" is any condition that would benefit from treatment of the present invention. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemia and lymphoid malignancies, in particular breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic, prostate or bladder cancer; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders. An exemplary disorder to be treated in accordance with the present invention is a solid, malignant tumor

The term "therapeutically effective amount" refers to an amount of a drug effective to treat a disease or disorder in a mammal. In the case of cancer, the therapeutically effective amount of the drug may: (i) reduce the number of cancer cells; (ii) reduce the tumor size; (iii) inhibit, retard, slow to some extent and preferably stop cancer cell infiltration into peripheral organs; (iv) inhibit (*i.e.*, slow to some extent and preferably stop) tumor metastasis; (v) inhibit tumor growth; and/or (vi) relieve to some extent one or more of the symptoms associated with the cancer. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. In animal models, efficacy may be assessed by physical measurements of the tumor during the course following administration of the ADC, and by determining partial and complete remission of tumor. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP) and/or determining the response rate (RR).

The term "bioavailability" refers to the systemic availability (*i.e.*, blood/plasma levels) of a given amount of drug administered to a patient. Bioavailability is an absolute term that indicates measurement of both

the time (rate) and total amount (extent) of drug that reaches the general circulation from an administered dosage form.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. A "tumor" comprises one or more cancerous cells.

5 Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (*e.g.*, epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer ("NSCLC"), adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, gastrointestinal stromal tumor (GIST),  
 10 pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, as well as head and neck cancer.

An "ErbB-expressing cancer" is one comprising cells which have ErbB protein present at their cell  
 15 surface. An "ErbB2-expressing cancer" is one which produces sufficient levels of ErbB2 at the surface of cells thereof, such that an anti-ErbB2 antibody can bind thereto and have a therapeutic effect with respect to the cancer.

A cancer which "overexpresses" a receptor, *e.g.* an ErbB receptor, is one which has significantly higher levels of the receptor, such as ErbB2, at the cell surface thereof, compared to a noncancerous cell of the same  
 20 tissue type. Such overexpression may be caused by gene amplification or by increased transcription or translation. ErbB receptor overexpression may be determined in a diagnostic or prognostic assay by evaluating increased levels of the ErbB protein present on the surface of a cell (*e.g.*, via an immunohistochemistry assay; IHC). Alternatively, or additionally, one may measure levels of ErbB-encoding nucleic acid in the cell, *e.g.*, via  
 25 fluorescent in situ hybridization (FISH; see WO 98/45479), southern blotting, or polymerase chain reaction (PCR) techniques, such as real time quantitative PCR (RT-PCR). Overexpression of the ErbB ligand, may be determined diagnostically by evaluating levels of the ligand (or nucleic acid encoding it) in the patient, *e.g.*, in a tumor biopsy or by various diagnostic assays such as the IHC, FISH, southern blotting, PCR or in vivo assays described above. One may also study ErbB receptor overexpression by measuring shed antigen (*e.g.*, ErbB  
 30 extracellular domain) in a biological fluid such as serum (see, *e.g.*, US 4933294; WO 91/05264; US 5401638; and Sias et al (1990) J. Immunol. Methods 132: 73-80). Aside from the above assays, various other in vivo assays are available to the skilled practitioner. For example, one may expose cells within the body of the patient to an antibody which is optionally labeled with a detectable label, *e.g.*, a radioactive isotope, and binding of the antibody to cells in the patient can be evaluated, *e.g.*, by external scanning for radioactivity or by analyzing a biopsy taken from a patient previously exposed to the antibody.

35 The tumors overexpressing HER2 are rated by immunohistochemical scores corresponding to the number of copies of HER2 molecules expressed per cell, and can be determined biochemically: 0 = 0-10,000 copies/cell, 1+ = at least about 200,000 copies/cell, 2+ = at least about 500,000 copies/cell, 3+ = about  $1-2 \times 10^6$  copies/cell. Overexpression of HER2 at the 3+ level, which leads to ligand-independent activation of the tyrosine kinase (Hudziak et al., (1987) Proc. Natl. Acad. Sci. USA 84:7159-7163), occurs in approximately 30%  
 40 of breast cancers, and in these patients, relapse-free survival and overall survival are diminished (Slamon et al

(1989) Science 244:707-712; Slamon et al (1987) Science, 235:177-182). Conversely, a cancer which is "not characterized by overexpression of the ErbB2 receptor" is one which, in a diagnostic assay, does not express higher than normal levels of ErbB2 receptor compared to a noncancerous cell of the same tissue type. The murine monoclonal anti-HER2 antibody inhibits the growth of breast cancer cell lines that overexpress HER2 at the 2+ and 3+ ( $1-2 \times 10^6$  HER2 receptors per cell) level, but has no activity on cells that express lower levels of HER2 (Lewis et al (1993) Cancer Immunol. Immunother. 37:255-263). Based on this observation, antibody 4D5 was humanized (huMAb4D5-8, rhuMAb HER2, US 5821337; Carter et al (1992) Proc. Natl. Acad. Sci. USA 89: 4285-4289) and tested in breast cancer patients whose tumors overexpress HER2 but who had progressed after conventional chemotherapy (Cobleigh et al (1999) J. Clin. Oncol. 17: 2639-2648). Most patient tumors in this trial expressed HER2 at the 3+ level, though a fraction was 2+.

A "hormone independent" cancer is one in which proliferation thereof is not dependent on the presence of a hormone which binds to a receptor expressed by cells in the cancer. Such cancers do not undergo clinical regression upon administration of pharmacological or surgical strategies that reduce the hormone concentration in or near the tumor. Examples of hormone independent cancers include androgen independent prostate cancer, estrogen independent breast cancer, endometrial cancer and ovarian cancer. Such cancers may begin as hormone dependent tumors and progress from a hormone-sensitive stage to a hormone-refractory tumor following anti-hormonal therapy.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.*,  $^{211}\text{At}$ ,  $^{131}\text{I}$ ,  $^{125}\text{I}$ ,  $^{90}\text{Y}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{153}\text{Sm}$ ,  $^{212}\text{Bi}$ ,  $^{32}\text{P}$ ,  $^{60}\text{Co}$ , and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including synthetic analogs and derivatives thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include Erlotinib (TARCEVA®, Genentech/OSI Pharm.), Bortezomib (VELCADE®, Millenium Pharm.), Fulvestrant (FASLODEX®, Astrazeneca), Sunitinib (SU11248, Pfizer), Letrozole (FEMARA®, Novartis), Imatinib mesylate (GLEEVEC®, Novartis), PTK787/ZK 222584 (Novartis), Oxaliplatin (Eloxatin®, Sanofi), 5-FU (5-fluorouracil), Leucovorin, Rapamycin (Sirolimus, RAPAMUNE®, Wyeth), Lapatinib (GSK572016, GlaxoSmithKline), Lonafermin (SCH 66336), Sorafenib (BAY43-9006, Bayer Labs.), and Gefitinib (IRESSA®, Astrazeneca), AG1478, AG1571 (SU 5271; Sugen), alkylating agents such as thiotepa and CYTOXAN® cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylmelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatins; calystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (*e.g.*,

calicheamicin, especially calicheamicin gammaII and calicheamicin omegaII (Angew Chem Intl. Ed. Engl. (1994) 33:183-186); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, anthramycin, azaserine, bleomycins, cactinomycin, carabycin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitostanol, mepitostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepe; taxoids, e.g., TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumburg, Illinois), and TAXOTERE® doxetaxel (Rhône-Poulenc Rorer, Antony, France); chloranbucil; GEMZAR® gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE® vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

Also included in this definition of "chemotherapeutic agent" are: (i) anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON® toremifene; (ii) aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestane, fadrozole, RIVISOR® vorozole, FEMARA® letrozole, and ARIMIDEX® anastrozole; (iii) anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); (iv) aromatase inhibitors; (v) protein kinase inhibitors; (vi) lipid kinase

inhibitors; (vii) antisense oligonucleotides, particularly those which inhibit expression of genes in signaling pathways implicated in aberrant cell proliferation, such as, for example, PKC-alpha, Ralf and H-Ras; (viii) ribozymes such as a VEGF expression inhibitor (e.g., ANGIOZYME® ribozyme) and a HER2 expression inhibitor; (ix) vaccines such as gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; PROLEUKIN® rIL-2; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmrH; (x) anti-angiogenic agents such as bevacizumab (AVASTIN®, Genentech); and (xi) pharmaceutically acceptable salts, acids or derivatives of any of the above.

Protein kinase inhibitors include tyrosine kinase inhibitors which inhibit to some extent tyrosine kinase activity of a tyrosine kinase such as an ErbB receptor. Examples of tyrosine kinase inhibitors include EGFR-targeted drugs such as: (i) antibodies which bind to EGFR, including MAb 579 (ATCC CRL HB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, US 4943533, Mendelsohn *et al.*) and variants thereof, such as chimerized 225 (C225 or Cetuximab; ERBITUX®, Imclone) and reshaped human 225 (H225) (WO 96/40210, Imclone Systems Inc.); antibodies that bind type II mutant EGFR (US 5212290); humanized and chimeric antibodies that bind EGFR (US 5891996); and human antibodies that bind EGFR, such as ABX-EGF (WO 98/50433); (ii) anti-EGFR antibody conjugated with a cytotoxic agent (EP 659439A2); and small molecules that bind to EGFR including ZD1839 or Gefitinib (IRESSA™; Astra Zeneca), Erlotinib HCl (CP-358774, TARCEVA™; Genentech/OSI) and AG1478, AG1571 (SU 5271; Sugen), quinazolines such as PD 153035, 4-(3-chloroanilino) quinazoline, pyridopyrimidines, pyrimidopyrimidines, pyrrolopyrimidines, such as CGP 59326, CGP 60261 and CGP 62706, and pyrazolopyrimidines, 4-(phenylamino)-7H-pyrrolo[2,3-d] pyrimidines, curcumin (diferuloyl methane, 4,5-bis (4-fluoroanilino)phthalimide), typhostines containing nitrothiophene moieties; PD-0183805 (Warner-Lambert); antisense molecules (e.g., those that bind to ErbB-encoding nucleic acid); quinoxalines (US 5804396); tryphostins (US 5804396); ZD6474 (Astra Zeneca); PTK-787 (Novartis/Schering AG); pan-ErbB inhibitors such as CI-1033 (Pfizer); Affinitac (ISIS 3521; Isis/Lilly); Imatinib mesylate (Gleevec; Novartis); PKI 166 (Novartis); GW2016 (Glaxo SmithKline); CI-1033 (Pfizer); EKB-569 (Wyeth); Semaxanib (Sugen); ZD6474 (AstraZeneca); PTK-787 (Novartis/Schering AG); INC-1C11 (Imclone); or as described in: US 5804396; WO 99/09016 (American Cyanamid); WO 98/43960 (American Cyanamid); WO 97/38983 (Warner Lambert); WO 99/06378 (Warner Lambert); WO 99/06396 (Warner Lambert); WO 96/30347 (Pfizer, Inc); WO 96/33978 (Zeneca); WO 96/3397 (Zeneca); and WO 96/33980 (Zeneca).

An "anti-angiogenic agent" refers to a compound which blocks, or interferes with to some degree, the development of blood vessels. The anti-angiogenic factor may, for instance, be a small molecule or antibody that binds to a growth factor or growth factor receptor involved in promoting angiogenesis. An exemplary anti-angiogenic agent is an antibody that binds to Vascular Endothelial Growth Factor (VEGF) such as bevacizumab (AVASTIN®, Genentech).

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor;

prolactin; placental lactogen; tumor necrosis factor- $\alpha$  and - $\beta$ ; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF- $\beta$ ; platelet-growth factor; transforming growth factors (TGFs) such as TGF- $\alpha$  and TGF- $\beta$ ; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon- $\alpha$ , - $\beta$ , and - $\gamma$ ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1 $\alpha$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; a tumor necrosis factor such as TNF- $\alpha$  or TNF- $\beta$ ; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" Biochemical Society Transactions, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs,  $\beta$ -lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

A "cardioprotectant" is a compound or composition which prevents or reduces myocardial dysfunction (*i.e.*, cardiomyopathy and/or congestive heart failure) associated with administration of a drug, such as an anthracycline antibiotic and/or an anti-ErbB2 antibody, to a patient. The cardioprotectant may, for example, block or reduce a free-radical-mediated cardiotoxic effect and/or prevent or reduce oxidative-stress injury. Examples of cardioprotectants encompassed by the present definition include the iron-chelating agent dexrazoxane (ICRF-187) (Seifert et al., *The Annals of Pharmacotherapy*, 28:1063-1072 (1994)); a lipid-lowering agent and/or anti-oxidant such as probucol (Singal et al., *J. Mol. Cell Cardiol.*, 27:1055-1063 (1995)); amifostine (aminothiols 2-[(3-aminopropyl)amino]ethanethiol-dihydrogen phosphate ester, also called WR-2721, and the dephosphorylated cellular uptake form thereof called WR-1065) and S-3-(3-methylaminopropylamino)propylphosphorothioic acid (WR-151327), see Green et al., (1994) Cancer Research,

- 54:738-741; digoxin (Bristow, M.R. ed. (1980) *Drug-Induced Heart Disease*. New York: Elsevier 191-215); beta-blockers such as metoprolol (Hjalmarson et al (1994) *Drugs* 47:Suppl 4:31-9; and Shaddy et al (1995) *Am. Heart J.*, 129:197-9); vitamin E; ascorbic acid (vitamin C); free radical scavengers such as oleanolic acid, ursolic acid and N-acetylcysteine (NAC); spin trapping compounds such as alpha-phenyl-tert-butyl nitron (PBN); (Paracchini et al (1993) *Anticancer Res.*, 13:1607-1612); selenoorganic compounds such as P251 (Elbesen); and the like.

10 An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the antibody nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the antibody where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

"Alkyl" is C<sub>1</sub>-C<sub>18</sub> hydrocarbon containing normal, secondary, tertiary or cyclic carbon atoms.

- 15 Examples of alkyl radicals include C<sub>1</sub>-C<sub>8</sub> hydrocarbon moieties such as, but not limited to: methyl (Me, -CH<sub>3</sub>), ethyl (Et, -CH<sub>2</sub>CH<sub>3</sub>), 1-propyl (n-Pr, n-propyl, -CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 2-propyl (i-Pr, i-propyl, -CH(CH<sub>3</sub>)<sub>2</sub>), 1-butyl (n-Bu, n-butyl, -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 2-methyl-1-propyl (i-Bu, i-butyl, -CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 2-butyl (s-Bu, s-butyl, -CH(CH<sub>3</sub>)CH<sub>2</sub>CH<sub>3</sub>), 2-methyl-2-propyl (t-Bu, t-butyl, -C(CH<sub>3</sub>)<sub>3</sub>), 1-pentyl (n-pentyl, -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 2-pentyl (-CH(CH<sub>3</sub>)CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 3-pentyl (-CH(CH<sub>2</sub>CH<sub>3</sub>)<sub>2</sub>), 2-methyl-2-butyl (-C(CH<sub>3</sub>)<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 3-methyl-2-butyl (-CH(CH<sub>3</sub>)CH(CH<sub>3</sub>)<sub>2</sub>), 3-methyl-1-butyl (-CH<sub>2</sub>CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 2-methyl-1-butyl (-CH<sub>2</sub>CH(CH<sub>3</sub>)CH<sub>2</sub>CH<sub>3</sub>), 1-hexyl (-CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 2-hexyl (-CH(CH<sub>3</sub>)CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 3-hexyl (-CH(CH<sub>2</sub>CH<sub>3</sub>)(CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>)), 2-methyl-2-pentyl (-C(CH<sub>3</sub>)<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 3-methyl-2-pentyl (-CH(CH<sub>3</sub>)CH(CH<sub>3</sub>)CH<sub>2</sub>CH<sub>3</sub>), 4-methyl-2-pentyl (-CH(CH<sub>3</sub>)CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 3-methyl-3-pentyl (-C(CH<sub>3</sub>)(CH<sub>2</sub>CH<sub>3</sub>)<sub>2</sub>), 2-methyl-3-pentyl (-CH(CH<sub>2</sub>CH<sub>3</sub>)CH(CH<sub>3</sub>)<sub>2</sub>), 2,3-dimethyl-2-butyl (-C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 3,3-dimethyl-2-butyl (-CH(CH<sub>3</sub>)C(CH<sub>3</sub>)<sub>3</sub>).

- 30 "Alkenyl" is C<sub>2</sub>-C<sub>18</sub> hydrocarbon containing normal, secondary, tertiary or cyclic carbon atoms with at least one site of unsaturation, i.e. a carbon-carbon, *sp*<sup>2</sup> double bond. Examples of alkenyl radicals include C<sub>2</sub>-C<sub>8</sub> hydrocarbon moieties such as, but not limited to: ethylene or vinyl (-CH=CH<sub>2</sub>), allyl (-CH<sub>2</sub>CH=CH<sub>2</sub>), cyclopentenyl (-C<sub>5</sub>H<sub>7</sub>), and 5-hexenyl (-CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH=CH<sub>2</sub>).

"Alkynyl" is C<sub>2</sub>-C<sub>18</sub> hydrocarbon containing normal, secondary, tertiary or cyclic carbon atoms with at least one site of unsaturation, i.e. a carbon-carbon, *sp* triple bond. Examples of alkynyl radicals include C<sub>2</sub>-C<sub>8</sub> hydrocarbon moieties such as, but not limited to: acetylenic (-C≡CH) and propargyl (-CH<sub>2</sub>C≡CH),

- 35 "Alkylene" refers to a saturated, branched or straight chain or cyclic hydrocarbon radical of 1-18 carbon atoms, and having two monovalent radical centers derived by the removal of two hydrogen atoms from the same or two different carbon atoms of a parent alkane. Typical alkylene radicals include C<sub>1</sub>-C<sub>8</sub> hydrocarbon moieties such

as, but are not limited to: methylene ( $-\text{CH}_2-$ ), 1,2-ethyl ( $-\text{CH}_2\text{CH}_2-$ ), 1,3-propyl ( $-\text{CH}_2\text{CH}_2\text{CH}_2-$ ), 1,4-butyl ( $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-$ ), and the like.

"Alkenylene" refers to an unsaturated, branched or straight chain or cyclic hydrocarbon radical of 2-18 carbon atoms, and having two monovalent radical centers derived by the removal of two hydrogen atoms from the same or two different carbon atoms of a parent alkene. Typical alkenylene radicals include  $\text{C}_2$ - $\text{C}_8$  hydrocarbon moieties such as, but are not limited to: 1,2-ethylene ( $-\text{CH}=\text{CH}-$ ).

"Alkynylene" refers to an unsaturated, branched or straight chain or cyclic hydrocarbon radical of 2-18 carbon atoms, and having two monovalent radical centers derived by the removal of two hydrogen atoms from the same or two different carbon atoms of a parent alkyne. Typical alkynylene radicals include  $\text{C}_2$ - $\text{C}_8$  hydrocarbon moieties such as, but are not limited to: acetylene ( $-\text{C}\equiv\text{C}-$ ), propargyl ( $-\text{CH}_2\text{C}\equiv\text{C}-$ ), and 4-pentynyl ( $-\text{CH}_2\text{CH}_2\text{CH}_2\text{C}\equiv\text{C}-$ ).

"Aryl", alone or in combination, means a monovalent aromatic hydrocarbon radical of 6-20 carbon atoms derived by the removal of one hydrogen atom from a single carbon atom of a parent aromatic ring system. Aryl radical may contain one, two or three rings wherein such rings may be attached together in a pendent manner, e.g. biphenyl, or may be fused, e.g. naphthalene or anthracene. Some aryl groups are represented in the exemplary structures as "Ar". Typical aryl groups include  $\text{C}_6$ - $\text{C}_{12}$  hydrocarbon moieties such as, but are not limited to, radicals derived from benzene, substituted benzene, naphthalene, anthracene, biphenyl, and the like.

"Arylalkyl" refers to an acyclic alkyl radical in which one of the hydrogen atoms bonded to a carbon atom, typically a terminal or  $\text{sp}^3$  carbon atom, is replaced with an aryl radical. Typical arylalkyl groups include, but are not limited to, benzyl, 2-phenylethan-1-yl, 2-phenylethen-1-yl, naphthylmethyl, 2-naphthylethan-1-yl, 2-naphthylethen-1-yl, naphthobenzyl, 2-naphthophenylethan-1-yl and the like. The arylalkyl group comprises 6 to 20 carbon atoms, e.g. the alkyl moiety, including alkanyl, alkenyl or alkynyl groups, of the arylalkyl group is 1 to 6 carbon atoms and the aryl moiety is 5 to 14 carbon atoms.

"Heteroarylalkyl" refers to an acyclic alkyl radical in which one of the hydrogen atoms bonded to a carbon atom, typically a terminal or  $\text{sp}^3$  carbon atom, is replaced with a heteroaryl radical. Typical heteroarylalkyl groups include, but are not limited to, 2-benzimidazolylmethyl, 2-furylethyl, and the like. The heteroarylalkyl group comprises 6 to 20 carbon atoms, e.g. the alkyl moiety, including alkanyl, alkenyl or alkynyl groups, of the heteroarylalkyl group is 1 to 6 carbon atoms and the heteroaryl moiety is 5 to 14 carbon atoms and 1 to 3 heteroatoms selected from N, O, P, and S. The heteroaryl moiety of the heteroarylalkyl group may be a monocycle having 3 to 7 ring members (2 to 6 carbon atoms or a bicycle having 7 to 10 ring members (4 to 9 carbon atoms and 1 to 3 heteroatoms selected from N, O, P, and S), for example: a bicyclo [4,5], [5,5], [5,6], or [6,6] system.

Alkyl, alkenylene, aryl, arylalkyl, and heteroarylalkyl groups may be substituted where one or more hydrogen atoms are each independently replaced with a substituent. Typical substituents include, but are not limited to,  $-\text{X}$ ,  $-\text{R}$ ,  $-\text{O}^-$ ,  $-\text{OR}$ ,  $-\text{SR}$ ,  $-\text{S}^-$ ,  $-\text{NR}_2$ ,  $-\text{NR}_3$ ,  $=\text{NR}$ ,  $-\text{CX}_3$ ,  $-\text{CN}$ ,  $-\text{OCN}$ ,  $-\text{SCN}$ ,  $-\text{N}=\text{C}=\text{O}$ ,  $-\text{NCS}$ ,  $-\text{NO}$ ,  $-\text{NO}_2$ ,  $=\text{N}_2$ ,  $-\text{N}_3$ ,  $-\text{NC}(=\text{O})\text{R}$ ,  $-\text{C}(=\text{O})\text{R}$ ,  $-\text{C}(=\text{O})\text{NR}_2$ ,  $-\text{SO}_3^-$ ,  $-\text{SO}_3\text{H}$ ,  $-\text{S}(=\text{O})_2\text{R}$ ,  $-\text{OS}(=\text{O})_2\text{OR}$ ,  $-\text{S}(=\text{O})_2\text{NR}$ ,  $-\text{S}(=\text{O})\text{R}$ ,  $-\text{OP}(=\text{O})(\text{OR})_2$ ,  $-\text{P}(=\text{O})(\text{OR})_2$ ,  $-\text{PO}_3^-$ ,  $-\text{PO}_3\text{H}_2$ ,  $-\text{C}(=\text{O})\text{R}$ ,  $-\text{C}(=\text{O})\text{X}$ ,  $-\text{C}(=\text{S})\text{R}$ ,  $-\text{CO}_2\text{R}$ ,  $-\text{CO}_2^-$ ,  $-\text{C}(=\text{S})\text{OR}$ ,  $-\text{C}(=\text{O})\text{SR}$ ,  $-\text{C}(=\text{S})\text{SR}$ ,  $-\text{C}(=\text{O})\text{NR}_2$ ,  $-\text{C}(=\text{S})\text{NR}_2$ ,  $-\text{C}(=\text{NR})\text{NR}_2$ , where each X is independently a halogen: F, Cl,

Br, or I; and each R is independently H, C<sub>1</sub>-C<sub>18</sub> alkyl, C<sub>6</sub>-C<sub>20</sub> aryl, C<sub>3</sub>-C<sub>14</sub> heterocycle, protecting group or prodrug moiety. Alkylene, alkenylene, and alkynylene groups as described above may also be similarly substituted.

"Heteroaryl", also known as heterocycle or heterocyclyl, refers to a ring system radical in which one or more ring atoms is a heteroatom, e.g. nitrogen, oxygen, and sulfur. The heteroaryl radical comprises 5 to 14 carbon atoms and 1 to 3 heteroatoms selected from N, O, P, and S. A heteroaryl may be a monocycle having 3 to 7 ring members (2 to 6 carbon atoms and 1 to 3 heteroatoms selected from N, O, P, and S) or a bicycle having 7 to 10 ring members (4 to 9 carbon atoms and 1 to 3 heteroatoms selected from N, O, P, and S), for example: a bicyclo [4,5], [5,5], [5,6], or [6,6] system. Heteroaryl compounds are described in Paquette, Leo A.; "Principles of Modern Heterocyclic Chemistry" (W.A. Benjamin, New York, 1968), particularly Chapters 1, 3, 4, 6, 7, and 9; "The Chemistry of Heterocyclic Compounds, A series of Monographs" (John Wiley & Sons, New York, 1950 to present), in particular Volumes 13, 14, 16, 19, and 28; and J. Am. Chem. Soc. (1960) 82:5566.

"Linker" or "link" means a chemical moiety comprising a covalent bond or a chain of atoms that covalently attaches an antibody to a drug moiety. In various embodiments, a linker is specified as L. Linkers include a divalent radical such as an alkylene, an arylene, a heteroarylene, moieties such as:  $-(CR_2)_nO(CR_2)_n-$ , repeating units of alkyloxy (e.g. polyethylenoxy, PEG, polymethyleneoxy) and alkylamino (e.g. polyethyleneamino, Jeffamine<sup>TM</sup>); and diacid ester and amides including succinate, succinamide, diglycolate, malonate, and caproamide.

The term "chiral" refers to molecules which have the property of non-superimposability of the mirror image partner, while the term "achiral" refers to molecules which are superimposable on their mirror image partner.

The term "stereoisomers" refers to compounds which have identical chemical constitution, but differ with regard to the arrangement of the atoms or groups in space.

"Diastereomer" refers to a stereoisomer with two or more centers of chirality and whose molecules are not mirror images of one another. Diastereomers have different physical properties, e.g. melting points, boiling points, spectral properties, and reactivities. Mixtures of diastereomers may separate under high resolution analytical procedures such as electrophoresis and chromatography.

"Enantiomers" refer to two stereoisomers of a compound which are non-superimposable mirror images of one another.

Stereochemical definitions and conventions used herein generally follow S. P. Parker, Ed., *McGraw-Hill Dictionary of Chemical Terms* (1984) McGraw-Hill Book Company, New York; and Eliel, E. and Wilen, S., *Stereochemistry of Organic Compounds* (1994) John Wiley & Sons, Inc., New York. Many organic compounds exist in optically active forms, i.e., they have the ability to rotate the plane of plane-polarized light. In describing an optically active compound, the prefixes D and L, or R and S, are used to denote the absolute configuration of the molecule about its chiral center(s). The prefixes d and l or (+) and (-) are employed to designate the sign of rotation of plane-polarized light by the compound, with (-) or l meaning that the compound is levorotatory. A compound prefixed with (+) or d is dextrorotatory. For a given chemical structure, these stereoisomers are identical except that they are mirror images of one another. A specific stereoisomer may also be referred to as an enantiomer, and a mixture of such isomers is often called an enantiomeric mixture. A 50:50 mixture of enantiomers is referred to as a racemic mixture or a racemate, which may occur where there has been no stereoselection or stereospecificity in a chemical reaction or process. The terms "racemic mixture"

and "racemate" refer to an equimolar mixture of two enantiomeric species, devoid of optical activity.

The phrase "pharmaceutically acceptable salt," as used herein, refers to pharmaceutically acceptable organic or inorganic salts of an ADC. Exemplary salts include, but are not limited, to sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucuronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, *p*-toluenesulfonate, and pamoate (*i.e.*, 1,1'-methylene-bis-(2-hydroxy-3-naphthoate)) salts. A pharmaceutically acceptable salt may involve the inclusion of another molecule such as an acetate ion, a succinate ion or other counterion. The counterion may be any organic or inorganic moiety that stabilizes the charge on the parent compound. Furthermore, a pharmaceutically acceptable salt may have more than one charged atom in its structure. Instances where multiple charged atoms are part of the pharmaceutically acceptable salt can have multiple counter ions. Hence, a pharmaceutically acceptable salt can have one or more charged atoms and/or one or more counterion.

"Pharmaceutically acceptable solvate" refers to an association of one or more solvent molecules and an ADC. Examples of solvents that form pharmaceutically acceptable solvates include, but are not limited to, water, isopropanol, ethanol, methanol, DMSO, ethyl acetate, acetic acid, and ethanolamine.

#### ANTIBODY-DRUG CONJUGATES

The compounds of the invention include those with utility for anticancer activity. In particular, the compounds include an antibody conjugated, *i.e.* covalently attached by a linker, to a drug moiety where the drug when not conjugated to an antibody has a cytotoxic or cytostatic effect. The biological activity of the drug moiety is thus modulated by conjugation to an antibody. The antibody-drug conjugates (ADC) of the invention may selectively deliver an effective dose of a cytotoxic agent to tumor tissue whereby greater selectivity, *i.e.* a lower efficacious dose may be achieved.

In one embodiment, the bioavailability of the ADC, or an intracellular metabolite of the ADC, is improved in a mammal when compared to the corresponding maytansinoid compound alone. Also, the bioavailability of the ADC, or an intracellular metabolite of the ADC is improved in a mammal when compared to the corresponding antibody alone (antibody of the ADC, without the drug moiety or linker).

In one embodiment, the maytansinoid drug moiety of the ADC is not cleaved from the antibody until the antibody-drug conjugate binds to a cell-surface receptor or enters a cell with a cell-surface receptor specific for the antibody of the antibody-drug conjugate. The drug moiety may be cleaved from the antibody after the antibody-drug conjugate enters the cell. The maytansinoid drug moiety may be intracellularly cleaved in a mammal from the antibody of the compound, or an intracellular metabolite of the compound, by enzymatic action, hydrolysis, oxidation, or other mechanism. For example, and in no way meant to limit the invention to a particular mechanism of action, the sulfur atom of the maytansinoid drug moiety of the ADC may be oxidized to a sulfone or sulfoxide group. Protons on carbons bound to the sulfone and sulfoxide may be removed under general or enzymatic catalysis inside the cell and result in a beta-elimination fragmentation that cleaves and separates the drug moiety from the antibody of the ADC. Alternatively, other electron withdrawing groups such as amides in the linker, antibody or drug moiety may effect similar fragmentation/cleavage mechanisms inside a cell.

Antibody-drug conjugates (ADC) may be represented by Formula I:

Ab-(L-D)<sub>p</sub>

I

or a pharmaceutically acceptable salt or solvate thereof, wherein:

Ab is an antibody which binds to an ErbB receptor, or which binds to one or more tumor-associated antigens or cell-surface receptors selected from (1)-(36):

- 5 (1) BMPR1B (bone morphogenetic protein receptor-type IB, Genbank accession no. NM\_001203);
- (2) E16 (LAT1, SLC7A5, Genbank accession no. NM\_003486);
- (3) STEAP1 (six transmembrane epithelial antigen of prostate, Genbank accession no. NM\_012449);
- (4) 0772P (CA125, MUC16, Genbank accession no. AF361486);
- 10 (5) MPF (MPF, MSLN, SMR, megakaryocyte potentiating factor, mesothelin, Genbank accession no. NM\_005823);
- (6) Napi3b (NAPI-3B, NPTIb, SLC34A2, solute carrier family 34 (sodium phosphate), member 2, type II sodium-dependent phosphate transporter 3b, Genbank accession no. NM\_006424);
- (7) Sema 5b (FLJ10372, KIAA1445, Mm.42015, SEMA5B, SEMAG, Semaphorin 5b Hlog, sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short
- 15 cytoplasmic domain, (semaphorin) 5B, Genbank accession no. AB040878);
- (8) PSCA hlg (2700050C12Rik, C530008O16Rik, RIKEN cDNA 2700050C12, RIKEN cDNA 2700050C12 gene, Genbank accession no. AY358628);
- (9) ETBR (Endothelin type B receptor, Genbank accession no. AY275463);
- (10) MSG783 (RNF124, hypothetical protein FLJ20315, Genbank accession no. NM\_017763);
- 20 (11) STEAP2 (HGNC\_8639, IPCA-1, PCANAP1, STAMP1, STEAP2, STMP, prostate cancer associated gene 1, prostate cancer associated protein 1, six transmembrane epithelial antigen of prostate 2, six transmembrane prostate protein, Genbank accession no. AF455138);
- (12) TrpM4 (BR22450, FLJ20041, TRPM4, TRPM4B, transient receptor potential cation channel, subfamily M, member 4, Genbank accession no. NM\_017636);
- 25 (13) CRIPTO (CR, CR1, CRGF, CRIPTO, TDGF1, teratocarcinoma-derived growth factor, Genbank accession no. NP\_003203 or NM\_003212);
- (14) CD21 (CR2 (Complement receptor 2) or C3DR (C3d/Epstein Barr virus receptor) or Hs.73792 Genbank accession no. M26004);
- 30 (15) CD79b (CD79B, CD79β, IgB (immunoglobulin-associated beta), B29, Genbank accession no. NM\_000626);
- (16) FcRH2 (IFGP4, IRTA4, SPAP1A (SH2 domain containing phosphatase anchor protein 1a), SPAP1B, SPAP1C, Genbank accession no. NM\_030764);
- (17) HER2 (Genbank accession no. M11730);
- (18) NCA (Genbank accession no. M18728);
- 35 (19) MDP (Genbank accession no. BC017023);
- (20) IL20Rα (Genbank accession no. AF184971);
- (21) Brevican (Genbank accession no. AF229053);
- (22) EphB2R (Genbank accession no. NM\_004442);
- (23) ASLG659 (Genbank accession no. AX092328);

(24) PSCA (Genbank accession no. AJ297436);

(25) GEDA (Genbank accession no. AY260763;

(26) BAFF-R (B cell -activating factor receptor, BLyS receptor 3, BR3, NP\_443177.1);

(27) CD22 (B-cell receptor CD22-B isoform, NP-001762.1);

5 (28) CD79a (CD79A, CD79 $\alpha$ , immunoglobulin-associated alpha, a B cell-specific protein that covalently interacts with Ig beta (CD79B) and forms a complex on the surface with Ig M molecules, transduces a signal involved in B-cell differentiation, Genbank accession No. NP\_001774.1);

(29) CXCR5 (Burkitt's lymphoma receptor 1, a G protein-coupled receptor that is activated by the CXCL13 chemokine, functions in lymphocyte migration and humoral defense, plays a role in HIV-2 infection and perhaps development of AIDS, lymphoma, myeloma, and leukemia, Genbank accession No. NP\_001707.1);

(30) HLA-DOB (Beta subunit of MHC class II molecule (Ia antigen) that binds peptides and presents them to CD4+ T lymphocytes, Genbank accession No. NP\_002111.1);

15 (31) P2X5 (Purinergic receptor P2X ligand-gated ion channel 5, an ion channel gated by extracellular ATP, may be involved in synaptic transmission and neurogenesis, deficiency may contribute to the pathophysiology of idiopathic detrusor instability, Genbank accession No. NP\_002552.2);

(32) CD72 (B-cell differentiation antigen CD72, Lyb-2, Genbank accession No. NP\_001773.1);

(33) LY64 (Lymphocyte antigen 64 (RP105), type I membrane protein of the leucine rich repeat (LRR) family, regulates B-cell activation and apoptosis, loss of function is associated with increased disease activity in patients with systemic lupus erythematosus, Genbank accession No. NP\_005573.1);

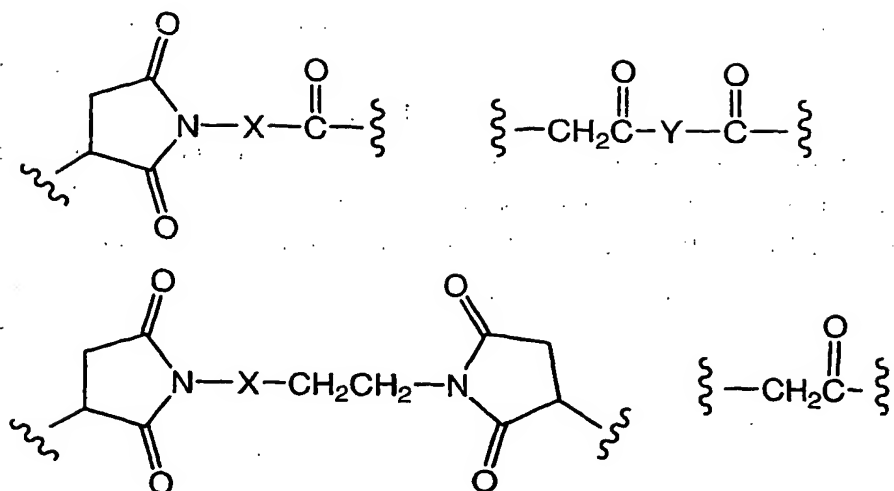
20 (34) FcRH1 (Fc receptor-like protein 1, a putative receptor for the immunoglobulin Fc domain that contains C2 type Ig-like and ITAM domains, may have a role in B-lymphocyte differentiation, Genbank accession No. NP\_443170.1);

(35) IRTA2 (Immunoglobulin superfamily receptor translocation associated 2, a putative immunoreceptor with possible roles in B cell development and lymphomagenesis; deregulation of the gene by translocation occurs in some B cell malignancies, Genbank accession No. NP\_112571.1); and

(36) TENB2 (putative transmembrane proteoglycan, related to the EGF/heregulin family of growth factors and follistatin, Genbank accession No. AF179274;

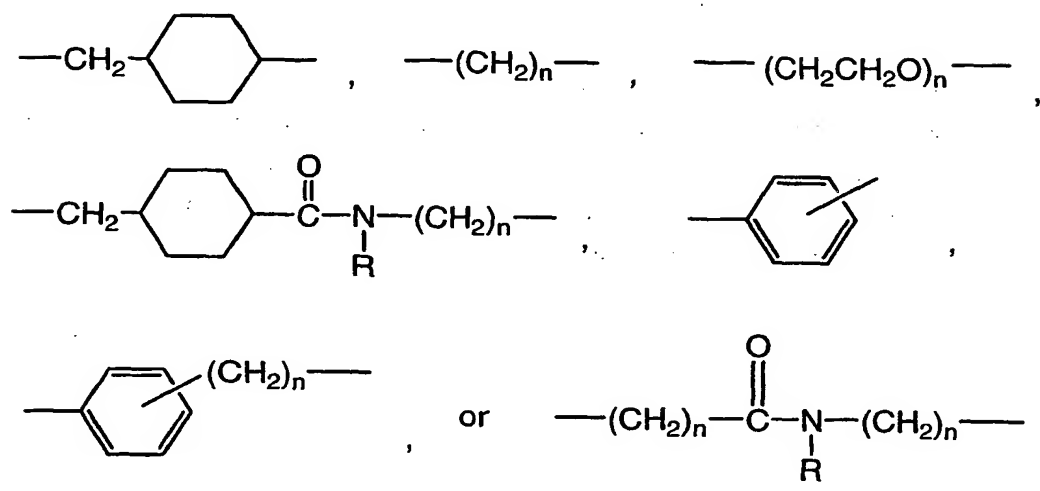
provided that the antibody is not TA.1.

30 L is a non-disulfide linker. L includes but is not limited to the structures:

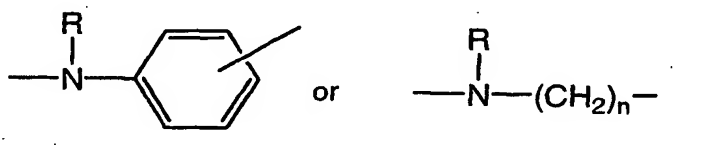


where the wavy lines indicate the covalent attachments to Ab and D;

X is:

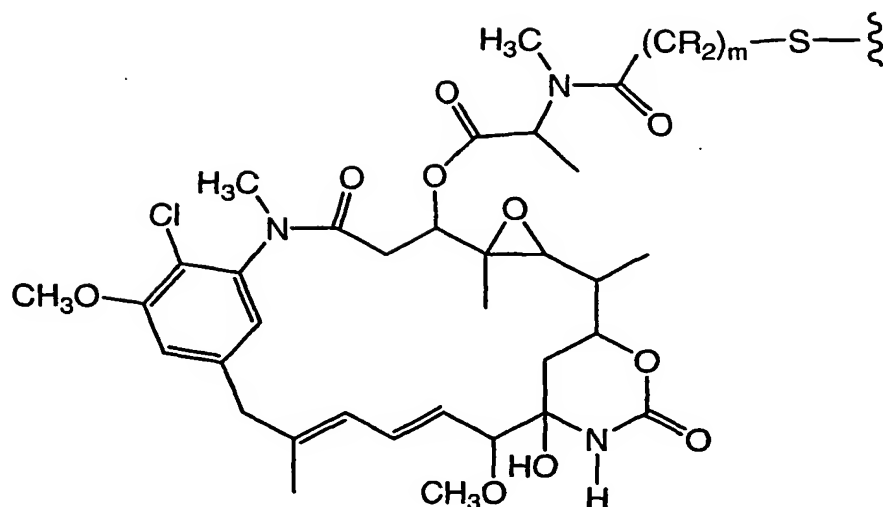


5 Y is:



R is independently H or  $\text{C}_1$ - $\text{C}_6$  alkyl; and n is 1 to 12;

D is a maytansinoid drug moiety. Maytansinoids include, but are not limited to the structure:



where the wavy line indicates the covalent attachment to L;

R is independently H or C<sub>1</sub>-C<sub>6</sub> alkyl; and

m is 1, 2, or 3.

- 5 The drug to antibody ratio or drug loading is represented by p for Formula I compounds. The drug loading value p is 1 to 8. Formula I compounds include all mixtures of variously loaded and attached antibody-drug conjugates where 1, 2, 3, 4, 5, 6, 7, and 8 drug moieties are covalently attached to the antibody.

In another embodiment, Ab is an antibody which binds to one or more tumor-associated antigens or cell-surface receptors selected from (1)-(16) and (18)-(36), i.e. not to an ErbB receptor, including HER2.

## 10 ANTIBODIES

The antibody unit (Ab-) of Formula I includes within its scope any unit of an antibody that binds or reactively associates or complexes with a receptor, antigen or other receptive moiety associated with a given target-cell population. An antibody can be any protein or protein-like molecule that binds to, complexes with, or reacts with a moiety of a cell population sought to be therapeutically or otherwise biologically modified. In one aspect, the antibody unit acts to deliver the maytansinoid drug moiety to the particular target cell population with which the antibody unit reacts. Such antibodies include, but are not limited to, large molecular weight proteins such as, full-length antibodies and antibody fragments.

Antibodies comprising the antibody-drug conjugates of the invention preferably retain the antigen binding capability of their native, wild type counterparts. Thus, antibodies of the invention are capable of binding, preferably specifically, to antigens. Such antigens include, for example, tumor-associated antigens (TAA), cell surface receptor proteins and other cell surface molecules, cell survival regulatory factors, cell proliferation regulatory factors, molecules associated with (for e.g., known or suspected to contribute functionally to) tissue development or differentiation, lymphokines, cytokines, molecules involved in cell cycle regulation, molecules involved in vasculogenesis and molecules associated with (for e.g., known or suspected to contribute functionally to) angiogenesis. The tumor-associated antigen may be a cluster differentiation factor (i.e., a CD protein). An antigen to which an antibody of the invention is capable of binding may be a member of a subset of one of the above-mentioned categories, wherein the other subset(s) of said category comprise other molecules/antigens that have a distinct characteristic (with respect to the antigen of interest).

In one embodiment, the antibody of the antibody-drug conjugates (ADC) specifically binds to a receptor encoded by an ErbB gene. The antibody may bind specifically to an ErbB receptor selected from EGFR, HER2, HER3 and HER4. The ADC may specifically bind to the extracellular domain (ECD) of the HER2 receptor and inhibit the growth of tumor cells which overexpress HER2 receptor. The antibody of the ADC may be a monoclonal antibody, e.g. a murine monoclonal antibody, a chimeric antibody, or a humanized antibody. A humanized antibody may be huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 or huMAb4D5-8 (trastuzumab). The antibody may be an antibody fragment, e.g. a Fab fragment.

Antibodies in Formula I antibody-drug conjugates (ADC) and which may be useful in the treatment of cancer include, but are not limited to, antibodies against cell surface receptors and tumor-associated antigens (TAA). Such tumor-associated antigens are known in the art, and can be prepared for use in generating antibodies using methods and information which are well known in the art. In attempts to discover effective cellular targets for cancer diagnosis and therapy, researchers have sought to identify transmembrane or otherwise tumor-associated polypeptides that are specifically expressed on the surface of one or more particular type(s) of cancer cell as compared to on one or more normal non-cancerous cell(s). Often, such tumor-associated polypeptides are more abundantly expressed on the surface of the cancer cells as compared to on the surface of the non-cancerous cells. The identification of such tumor-associated cell surface antigen polypeptides has given rise to the ability to specifically target cancer cells for destruction via antibody-based therapies.

Examples of TAA include, but are not limited to, Tumor-Associated Antigens (1)-(36) listed below. For convenience, information relating to these antigens, all of which are known in the art, is listed below and includes names, alternative names, Genbank accession numbers and primary reference(s), following nucleic acid and protein sequence identification conventions of the National Center for Biotechnology Information (NCBI). Nucleic acid and protein sequences corresponding to TAA (1)-(36) are available in public databases such as GenBank. Tumor-associated antigens targeted by antibodies include all amino acid sequence variants and isoforms possessing at least about 70%, 80%, 85%, 90%, or 95% sequence identity relative to the sequences identified in the cited references, or which exhibit substantially the same biological properties or characteristics as a TAA having a sequence found in the cited references. For example, a TAA having a variant sequence generally is able to bind specifically to an antibody that binds specifically to the TAA with the corresponding sequence listed. The sequences and disclosure in the reference specifically recited herein are expressly incorporated by reference.

#### TUMOR-ASSOCIATED ANTIGENS (1)-(36):

(1) BMPRI1B (bone morphogenetic protein receptor-type IB, Genbank accession no. NM\_001203)

ten Dijke, P., et al Science 264 (5155):101-104 (1994), Oncogene 14 (11):1377-1382 (1997));

WO2004063362 (Claim 2); WO2003042661 (Claim 12); US2003134790-A1 (Page 38-39);

WO2002102235 (Claim 13; Page 296); WO2003055443 (Page 91-92); WO200299122 (Example 2;

Page 528-530); WO2003029421 (Claim 6); WO2003024392 (Claim 2; Fig 112); WO200298358

(Claim 1; Page 183); WO200254940 (Page 100-101); WO200259377 (Page 349-350);

WO200230268 (Claim 27; Page 376); WO200148204 (Example; Fig 4)

NP\_001194 bone morphogenetic protein receptor, type IB /pid=NP\_001194.1 -

Cross-references: MIM:603248; NP\_001194.1; AY065994

(2) E16 (LAT1, SLC7A5, Genbank accession no. NM\_003486)

Biochem. Biophys. Res. Commun. 255 (2), 283-288 (1999), Nature 395 (6699):288-291 (1998), Gaugitsch, H.W., et al (1992) J. Biol. Chem. 267 (16):11267-11273; WO2004048938 (Example 2); WO2004032842 (Example IV); WO2003042661 (Claim 12); WO2003016475 (Claim 1); WO200278524 (Example 2); WO200299074 (Claim 19; Page 127-129); WO200286443 (Claim 27; Pages 222, 393); WO2003003906 (Claim 10; Page 293); WO200264798 (Claim 33; Page 93-95); WO200014228 (Claim 5; Page 133-136); US2003224454 (Fig 3); WO2003025138 (Claim 12; Page 150); US 20050107595; US 20050106644; NP\_003477 solute carrier family 7 (cationic amino acid transporter, y+

system), member 5 /pid=NP\_003477.3 - Homo sapiens

Cross-references: MIM:600182; NP\_003477.3; NM\_015923; NM\_003486\_1

(3) STEAP1 (six transmembrane epithelial antigen of prostate, Genbank accession no. NM\_012449)

Cancer Res. 61 (15), 5857-5860 (2001), Hubert, R.S., et al (1999) Proc. Natl. Acad. Sci. U.S.A. 96 (25):14523-14528; WO2004065577 (Claim 6); WO2004027049 (Fig 1L); EP1394274 (Example 11); WO2004016225 (Claim 2); WO2003042661 (Claim 12); US2003157089 (Example 5); US2003185830 (Example 5); US2003064397 (Fig 2); WO200289747 (Example 5; Page 618-619); WO2003022995 (Example 9; Fig 13A, Example 53; Page 173, Example 2; Fig 2A);

NP\_036581 six transmembrane epithelial antigen of the prostate

Cross-references: MIM:604415; NP\_036581.1; NM\_012449\_1

(4) 0772P (CA125, MUC16, Genbank accession no. AF361486)

J. Biol. Chem. 276 (29):27371-27375 (2001); WO2004045553 (Claim 14); WO200292836 (Claim 6; Fig 12); WO200283866 (Claim 15; Page 116-121); US2003124140 (Example 16); US2003091580 (Claim 6); WO200206317 (Claim 6; Page 400-408);

Cross-references: GI:34501467; AAK74120.3; AF361486\_1

(5) MPF (MPF, MSLN, SMR, megakaryocyte potentiating factor, mesothelin, Genbank accession no. NM\_005823)

Yamaguchi, N., et al Biol. Chem. 269 (2), 805-808 (1994), Proc. Natl. Acad. Sci. U.S.A. 96 (20):11531-11536 (1999), Proc. Natl. Acad. Sci. U.S.A. 93 (1):136-140 (1996), J. Biol. Chem. 270 (37):21984-21990 (1995); WO2003101283 (Claim 14); (WO2002102235 (Claim 13; Page 287-288); WO2002101075 (Claim 4; Page 308-309); WO200271928 (Page 320-321); WO9410312 (Page 52-57);

Cross-references: MIM:601051; NP\_005814.2; NM\_005823\_1

(6) Napi3b (NAPI-3B, NPTIb, SLC34A2, solute carrier family 34 (sodium phosphate), member 2, type II sodium-dependent phosphate transporter 3b, Genbank accession no. NM\_006424)

J. Biol. Chem. 277 (22):19665-19672 (2002), Genomics 62 (2):281-284 (1999), Feild, J.A., et al (1999)

Biochem. Biophys. Res. Commun. 258 (3):578-582; WO2004022778 (Claim 2); EP1394274 (Example 11);

WO2002102235 (Claim 13; Page 326); EP875569 (Claim 1; Page 17-19); WO200157188 (Claim 20; Page 329); WO2004032842 (Example IV); WO200175177 (Claim 24; Page 139-140);  
Cross-references: MIM:604217; NP\_006415.1; NM\_006424\_1

- 5 (7) Sema 5b (FLJ10372, KIAA1445, Mm.42015, SEMA5B, SEMAG; Semaphorin 5b Hlog, sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5B, Genbank accession no. AB040878)  
Nagase T., et al (2000) DNA Res. 7 (2):143-150; WO2004000997 (Claim 1); WO2003003984 (Claim 1);  
WO200206339 (Claim 1; Page 50); WO200188133 (Claim 1; Page 41-43, 48-58); WO2003054152 (Claim 20);  
10 WO2003101400 (Claim 11);  
Accession: Q9P283; EMBL; AB040878; BAA95969.1. Genew; HGNC:10737;
- (8) PSCA hlg (2700050C12Rik, C530008O16Rik, RIKEN cDNA 2700050C12, RIKEN cDNA  
2700050C12 gene, Genbank accession no. AY358628); Ross et al (2002) Cancer Res. 62:2546-2553;  
15 US2003129192 (Claim 2); US2004044180 (Claim 12); US2004044179 (Claim 11); US2003096961 (Claim  
11); US2003232056 (Example 5); WO2003105758 (Claim 12); US2003206918 (Example 5); EP1347046  
(Claim 1); WO2003025148 (Claim 20);  
Cross-references: GI:37182378; AAQ88991.1; AY358628\_1
- 20 (9) ETBR (Endothelin type B receptor, Genbank accession no. AY275463);  
Nakamuta M., et al Biochem. Biophys. Res. Commun. 177, 34-39, 1991; Ogawa Y., et al Biochem. Biophys.  
Res. Commun. 178, 248-255, 1991; Arai H., et al Jpn. Circ. J. 56, 1303-1307, 1992; Arai H., et al J. Biol. Chem.  
268, 3463-3470, 1993; Sakamoto A., Yanagisawa M., et al Biochem. Biophys. Res. Commun. 178, 656-663,  
1991; Elshourbagy N.A., et al J. Biol. Chem. 268, 3873-3879, 1993; Haendler B., et al J. Cardiovasc.  
25 Pharmacol. 20, s1-S4, 1992; Tsutsumi M., et al Gene 228, 43-49, 1999; Strausberg R.L., et al Proc. Natl. Acad.  
Sci. U.S.A. 99, 16899-16903, 2002; Bourgeois C., et al J. Clin. Endocrinol. Metab. 82, 3116-3123, 1997;  
Okamoto Y., et al Biol. Chem. 272, 21589-21596, 1997; Verheij J.B., et al Am. J. Med. Genet. 108, 223-225,  
2002; Hofstra R.M.W., et al Eur. J. Hum. Genet. 5, 180-185, 1997; Puffenberger E.G., et al Cell 79, 1257-1266,  
1994; Attie T., et al, Hum. Mol. Genet. 4, 2407-2409, 1995; Auricchio A., et al Hum. Mol. Genet. 5:351-354,  
30 1996; Amiel J., et al Hum. Mol. Genet. 5, 355-357, 1996; Hofstra R.M.W., et al Nat. Genet. 12, 445-447, 1996;  
Svensson P.J., et al Hum. Genet. 103, 145-148, 1998; Fuchs S., et al Mol. Med. 7, 115-124, 2001; Pingault V.,  
et al (2002) Hum. Genet. 111, 198-206; WO2004045516 (Claim 1); WO2004048938 (Example 2);  
WO2004040000 (Claim 151); WO2003087768 (Claim 1); WO2003016475 (Claim 1); WO2003016475 (Claim  
1); WO200261087 (Fig 1); WO2003016494 (Fig 6); WO2003025138 (Claim 12; Page 144); WO200198351  
35 (Claim 1; Page 124-125); EP522868 (Claim 8; Fig 2); WO200177172 (Claim 1; Page 297-299);  
US2003109676; US6518404 (Fig 3); US5773223 (Claim 1a; Col 31-34); WO2004001004;
- (10) MSG783 (RNF124, hypothetical protein FLJ20315, Genbank accession no. NM\_017763);

WO2003104275 (Claim 1); WO2004046342 (Example 2); WO2003042661 (Claim 12); WO2003083074 (Claim 14; Page 61); WO2003018621 (Claim 1); WO2003024392 (Claim 2; Fig 93); WO200166689 (Example 6);

Cross-references: LocusID:54894; NP\_060233.2; NM\_017763\_1

5

(11) STEAP2 (HGNC\_8639, IPCA-1, PCANAP1, STAMP1, STEAP2, STMP, prostate cancer associated gene 1, prostate cancer associated protein 1, six transmembrane epithelial antigen of prostate 2, six transmembrane prostate protein, Genbank accession no. AF455138)

Lab. Invest. 82 (11):1573-1582 (2002)); WO2003087306; US2003064397 (Claim 1; Fig 1); WO200272596

10 (Claim 13; Page 54-55); WO200172962 (Claim 1; Fig 4B); WO2003104270 (Claim 11); WO2003104270 (Claim 16); US2004005598 (Claim 22); WO2003042661 (Claim 12); US2003060612 (Claim 12; Fig 10); WO200226822 (Claim 23; Fig 2); WO200216429 (Claim 12; Fig 10);

Cross-references: GI:22655488; AAN04080.1; AF455138\_1

15 (12) TrpM4 (BR22450, FLJ20041, TRPM4, TRPM4B, transient receptor potential cation channel, subfamily M, member 4, Genbank accession no. NM\_017636)

Xu, X.Z., et al Proc. Natl. Acad. Sci. U.S.A. 98 (19):10692-10697 (2001), Cell 109 (3):397-407 (2002), J. Biol. Chem. 278 (33):30813-30820 (2003)); US2003143557 (Claim 4); WO200040614 (Claim 14; Page 100-103); WO200210382 (Claim 1; Fig 9A); WO2003042661 (Claim 12); WO200230268 (Claim 27; Page 391);

20 US2003219806 (Claim 4); WO200162794 (Claim 14; Fig 1A-D);

Cross-references: MIM:606936; NP\_060106.2; NM\_017636\_1

(13) CRIPTO (CR, CR1, CRGF, CRIPTO, TDGF1, teratocarcinoma-derived growth factor, Genbank accession no. NP\_003203 or NM\_003212)

25 Ciccodicola, A., et al EMBO J. 8 (7):1987-1991 (1989), Am. J. Hum. Genet. 49 (3):555-565 (1991)); US2003224411 (Claim 1); WO2003083041 (Example 1); WO2003034984 (Claim 12); WO200288170 (Claim 2; Page 52-53); WO2003024392 (Claim 2; Fig 58); WO200216413 (Claim 1; Page 94-95, 105);

WO200222808 (Claim 2; Fig 1); US5854399 (Example 2; Col 17-18); US5792616 (Fig 2);

Cross-references: MIM:187395; NP\_003203.1; NM\_003212\_1

30

(14) CD21 (CR2 (Complement receptor 2) or C3DR (C3d/Epstein Barr virus receptor) or Hs.73792 Genbank accession no. M26004)

Fujisaku et al (1989) J. Biol. Chem. 264 (4):2118-2125); Weis J.J., et al J. Exp. Med. 167, 1047-1066, 1988; Moore M., et al Proc. Natl. Acad. Sci. U.S.A. 84, 9194-9198, 1987; Barel M., et al Mol.

35 Immunol. 35, 1025-1031, 1998; Weis J.J., et al Proc. Natl. Acad. Sci. U.S.A. 83, 5639-5643, 1986; Sinha S.K., et al (1993) J. Immunol. 150, 5311-5320; WO2004045520 (Example 4); US2004005538 (Example 1); WO2003062401 (Claim 9); WO2004045520 (Example 4); WO9102536 (Fig 9.1-9.9);

WO2004020595 (Claim 1);

Accession: P20023; Q13866; Q14212; EMBL; M26004; AAA35786.1.

40

(15) CD79b (CD79B, CD79 $\beta$ , Igb (immunoglobulin-associated beta), B29, Genbank accession no. NM\_000626 or 11038674)

Proc. Natl. Acad. Sci. U.S.A. (2003) 100 (7):4126-4131, Blood (2002) 100 (9):3068-3076, Muller et al (1992) Eur. J. Immunol. 22 (6):1621-1625; WO2004016225 (claim 2, Fig 140); WO2003087768, US2004101874 (claim 1, page 102); WO2003062401 (claim 9); WO200278524 (Example 2); US2002150573 (claim 5, page 15); US5644033; WO2003048202 (claim 1, pages 306 and 309); WO 99/558658, US6534482 (claim 13, Fig 17A/B); WO200055351 (claim 11, pages 1145-1146);

Cross-references: MIM:147245; NP\_000617.1; NM\_000626\_1

- 10 (16) FcRH2 (IFGP4, IRTA4, SPAP1A (SH2 domain containing phosphatase anchor protein 1a), SPAP1B, SPAP1C, Genbank accession no. NM\_030764, AY358130)  
Genome Res. 13 (10):2265-2270 (2003), Immunogenetics 54 (2):87-95 (2002), Blood 99 (8):2662-2669 (2002), Proc. Natl. Acad. Sci. U.S.A. 98 (17):9772-9777 (2001), Xu, M.J., et al (2001) Biochem. Biophys. Res. Commun. 280 (3):768-775; WO2004016225 (Claim 2); WO2003077836; WO200138490 (Claim 5; Fig 18D-1-18D-2); WO2003097803 (Claim 12); WO2003089624 (Claim 25);  
15 Cross-references: MIM:606509; NP\_110391.2; NM\_030764\_1

(17) HER2 (ErbB2, Genbank accession no. M11730)

- 20 Coussens L., et al Science (1985) 230(4730):1132-1139; Yamamoto T., et al Nature 319, 230-234, 1986; Semba K., et al Proc. Natl. Acad. Sci. U.S.A. 82, 6497-6501, 1985; Swiercz J.M., et al J. Cell Biol. 165, 869-880, 2004; Kuhns J.J., et al J. Biol. Chem. 274, 36422-36427, 1999; Cho H.-S., et al Nature 421, 756-760, 2003; Ehsani A., et al (1993) Genomics 15, 426-429; WO2004048938 (Example 2); WO2004027049 (Fig 1I); WO2004009622; WO2003081210; WO2003089904 (Claim 9); WO2003016475 (Claim 1); US2003118592; WO2003008537 (Claim 1); WO2003055439  
25 (Claim 29; Fig 1A-B); WO2003025228 (Claim 37; Fig 5C); WO200222636 (Example 13; Page 95-107); WO200212341 (Claim 68; Fig 7); WO200213847 (Page 71-74); WO200214503 (Page 114-117); WO200153463 (Claim 2; Page 41-46); WO200141787 (Page 15); WO200044899 (Claim 52; Fig 7); WO200020579 (Claim 3; Fig 2); US5869445 (Claim 3; Col 31-38); WO9630514 (Claim 2; Page 56-61); EP1439393 (Claim 7); WO2004043361 (Claim 7); WO2004022709; WO200100244  
30 (Example 3; Fig 4);  
Accession: P04626; EMBL; M11767; AAA35808.1. EMBL; M11761; AAA35808.1.

(18) NCA (CEACAM6, Genbank accession no. M18728);

- 35 Barnett T., et al Genomics 3, 59-66, 1988; Tawaragi Y., et al Biochem. Biophys. Res. Commun. 150, 89-96, 1988; Strausberg R.L., et al Proc. Natl. Acad. Sci. U.S.A. 99:16899-16903, 2002; WO2004063709; EP1439393 (Claim 7); WO2004044178 (Example 4); WO2004031238; WO2003042661 (Claim 12); WO200278524 (Example 2); WO200286443 (Claim 27; Page 427); WO200260317 (Claim 2);  
Accession: P40199; Q14920; EMBL; M29541; AAA59915.1. EMBL; M18728;

- 40 (19) MDP (DPEP1, Genbank accession no. BC017023)

Proc. Natl. Acad. Sci. U.S.A. 99 (26):16899-16903 (2002)); WO2003016475 (Claim 1);  
 WO200264798 (Claim 33; Page 85-87); JP05003790 (Fig 6-8); WO9946284 (Fig 9);  
 Cross-references: MIM:179780; AAH17023.1; BC017023\_1

- 5 (20) IL20R $\alpha$  (IL20Ra, ZCYTOR7, Genbank accession no. AF184971);  
 Clark H.F., et al Genome Res. 13, 2265-2270, 2003; Mungall A.J., et al Nature 425, 805-811, 2003;  
 Blumberg H., et al Cell 104, 9-19, 2001; Dumoutier L., et al J. Immunol. 167, 3545-3549, 2001;  
 Parrish-Novak J., et al J. Biol. Chem. 277, 47517-47523, 2002; Pletnev S., et al (2003) Biochemistry  
 42:12617-12624; Sheikh F., et al (2004) J. Immunol. 172, 2006-2010; EP1394274 (Example 11);
- 10 US2004005320 (Example 5); WO2003029262 (Page 74-75); WO2003002717 (Claim 2; Page 63);  
 WO200222153 (Page 45-47); US2002042366 (Page 20-21); WO200146261 (Page 57-59);  
 WO200146232 (Page 63-65); WO9837193 (Claim 1; Page 55-59);  
 Accession: Q9UHF4; Q6UWA9; Q96SH8; EMBL; AF184971; AAF01320.1.
- 15 (21) Brevican (BCAN, BEHAB, Genbank accession no. AF229053)  
 Gary S.C., et al Gene 256, 139-147, 2000; Clark H.F., et al Genome Res. 13, 2265-2270, 2003;  
 Strausberg R.L., et al Proc. Natl. Acad. Sci. U.S.A. 99, 16899-16903, 2002; US2003186372 (Claim  
 11); US2003186373 (Claim 11); US2003119131 (Claim 1; Fig 52); US2003119122 (Claim 1; Fig  
 52); US2003119126 (Claim 1); US2003119121 (Claim 1; Fig 52); US2003119129 (Claim 1);
- 20 US2003119130 (Claim 1); US2003119128 (Claim 1; Fig 52); US2003119125 (Claim 1);  
 WO2003016475 (Claim 1); WO200202634 (Claim 1);
- (22) EphB2R (DRT, ERK, Hek5, EPHT3, Tyro5, Genbank accession no. NM\_004442)  
 Chan, J. and Watt, V.M., Oncogene 6 (6), 1057-1061 (1991) Oncogene 10 (5):897-905 (1995), Annu. Rev.  
 25 Neurosci. 21:309-345 (1998), Int. Rev. Cytol. 196:177-244 (2000)); WO2003042661 (Claim 12);  
 WO200053216 (Claim 1; Page 41); WO2004065576 (Claim 1); WO2004020583 (Claim 9); WO2003004529  
 (Page 128-132); WO200053216 (Claim 1; Page 42);  
 Cross-references: MIM:600997; NP\_004433.2; NM\_004442\_1
- 30 (23) ASLG659 (B7h, Genbank accession no. AX092328)  
 US20040101899 (Claim 2); WO2003104399 (Claim 11); WO2004000221 (Fig 3); US2003165504 (Claim 1);  
 US2003124140 (Example 2); US2003065143 (Fig 60); WO2002102235 (Claim 13; Page 299); US2003091580  
 (Example 2); WO200210187 (Claim 6; Fig 10); WO200194641 (Claim 12; Fig 7b); WO200202624 (Claim 13;  
 Fig 1A-1B); US2002034749 (Claim 54; Page 45-46); WO200206317 (Example 2; Page 320-321, Claim 34;  
 35 Page 321-322); WO200271928 (Page 468-469); WO200202587 (Example 1; Fig 1); WO200140269 (Example  
 3; Pages 190-192); WO200036107 (Example 2; Page 205-207); WO2004053079 (Claim 12); WO2003004989  
 (Claim 1); WO200271928 (Page 233-234, 452-453); WO 0116318;
- (24) PSCA (Prostate stem cell antigen precursor, Genbank accession no. AJ297436)

Reiter R.E., et al Proc. Natl. Acad. Sci. U.S.A. 95, 1735-1740, 1998; Gu Z., et al Oncogene 19, 1288-1296, 2000; Biochem. Biophys. Res. Commun. (2000) 275(3):783-788; WO2004022709; EP1394274 (Example 11); US2004018553 (Claim 17); WO2003008537 (Claim 1); WO200281646 (Claim 1; Page 164); WO2003003906 (Claim 10; Page 288); WO200140309 (Example 1; Fig 17);  
 5 US2001055751 (Example 1; Fig 1b); WO200032752 (Claim 18; Fig 1); WO9851805 (Claim 17; Page 97); WO9851824 (Claim 10; Page 94); WO9840403 (Claim 2; Fig 1B);  
 Accession: O43653; EMBL; AF043498; AAC39607.1.

(25) GEDA (Genbank accession No. AY260763);

10 AAP14954 lipoma HMGIC fusion-partner-like protein /pid=AAP14954.1 - Homo sapiens  
 Species: Homo sapiens (human)  
 WO2003054152 (Claim 20); WO2003000842 (Claim 1); WO2003023013 (Example 3, Claim 20);  
 US2003194704 (Claim 45);  
 Cross-references: GI:30102449; AAP14954.1; AY260763\_1

15 (26) BAFF-R (B cell -activating factor receptor, BLyS receptor 3, BR3, Genbank accession No. AF116456);  
 BAFF receptor /pid=NP\_443177.1 - Homo sapiens  
 Thompson, J.S., et al Science 293 (5537), 2108-2111 (2001); WO2004058309; WO2004011611;  
 WO2003045422 (Example; Page 32-33); WO2003014294 (Claim 35; Fig 6B); WO2003035846 (Claim 70;  
 20 Page 615-616); WO200294852 (Col 136-137); WO200238766 (Claim 3; Page 133); WO200224909 (Example  
 3; Fig 3);  
 Cross-references: MIM:606269; NP\_443177.1; NM\_052945\_1; AF132600

(27) CD22 (B-cell receptor CD22-B isoform, BL-CAM, Lyb-8, Lyb8, SIGLEC-2, FLJ22814, Genbank  
 25 accession No. AK026467);  
 Wilson et al (1991) J. Exp. Med. 173:137-146; WO2003072036 (Claim 1; Fig 1);  
 Cross-references: MIM:107266; NP\_001762.1; NM\_001771\_1

(28) CD79a (CD79A, CD79 $\alpha$ , immunoglobulin-associated alpha, a B cell-specific protein that covalently  
 30 interacts with Ig beta (CD79B) and forms a complex on the surface with Ig M molecules, transduces a signal  
 involved in B-cell differentiation) PROTEIN SEQUENCE Full mpggpgv...dvqlekp (1..226; 226 aa), pI: 4.84,  
 MW: 25028 TM: 2 [P] Gene Chromosome: 19q13.2, Genbank accession No. NP\_001774.10)  
 WO2003088808, US20030228319; WO2003062401 (claim 9); US2002150573 (claim 4, pages 13-14);  
 WO9958658 (claim 13, Fig 16); WO9207574 (Fig 1); US5644033; Ha et al (1992) J. Immunol. 148(5):1526-  
 35 1531; Mueller et al (1992) Eur. J. Biochem. 22:1621-1625; Hashimoto et al (1994) Immunogenetics 40(4):287-  
 295; Preud'homme et al (1992) Clin. Exp. Immunol. 90(1):141-146; Yu et al (1992) J. Immunol. 148(2) 633-  
 637; Sakaguchi et al (1988) EMBO J. 7(11):3457-3464;

(29) CXCR5 (Burkitt's lymphoma receptor 1, a G protein-coupled receptor that is activated by the CXCL13  
 40 chemokine, functions in lymphocyte migration and humoral defense, plays a role in HIV-2 infection and

perhaps development of AIDS, lymphoma, myeloma, and leukemia) PROTEIN SEQUENCE Full  
mnypltl...atstltf (1..372; 372 aa, pI: 8.54 MW: 41959 TM: 7 [P] Gene Chromosome: 11q23.3, Genbank  
accession No. NP\_001707.1)

WO2004040000; WO2004015426; US2003105292 (Example 2); US6555339 (Example 2); WO200261087

- 5 (Fig 1); WO200157188 (Claim 20, page 269); WO200172830 (pages 12-13); WO200022129 (Example 1,  
pages 152-153, Example 2, pages 254-256); WO9928468 (claim 1, page 38); US5440021 (Example 2, col 49-  
52); WO9428931 (pages 56-58); WO9217497 (claim 7, Fig 5); Dobner et al (1992) Eur. J. Immunol. 22:2795-  
2799; Barella et al (1995) Biochem. J. 309:773-779;

- 10 (30) HLA-DOB (Beta subunit of MHC class II molecule (Ia antigen) that binds peptides and presents them to  
CD4+ T lymphocytes) PROTEIN SEQUENCE Full mgsgwvp...vllpqsc (1..273; 273 aa, pI: 6.56 MW: 30820  
TM: 1 [P] Gene Chromosome: 6p21.3, Genbank accession No. NP\_002111.1)

Tonnelle et al (1985) EMBO J. 4(11):2839-2847; Jonsson et al (1989) Immunogenetics 29(6):411-413; Beck et  
al (1992) J. Mol. Biol. 228:433-441; Strausberg et al (2002) Proc. Natl. Acad. Sci USA 99:16899-16903;

- 15 Serenius et al (1987) J. Biol. Chem. 262:8759-8766; Beck et al (1996) J. Mol. Biol. 255:1-13; Naruse et al  
(2002) Tissue Antigens 59:512-519; WO9958658 (claim 13, Fig 15); US6153408 (Col 35-38); US5976551 (col  
168-170); US6011146 (col 145-146); Kasahara et al (1989) Immunogenetics 30(1):66-68; Larhammar et al  
(1985) J. Biol. Chem. 260(26):14111-14119;

- 20 (31) P2X5 (Purinergic receptor P2X ligand-gated ion channel 5, an ion channel gated by extracellular ATP, may  
be involved in synaptic transmission and neurogenesis, deficiency may contribute to the pathophysiology of  
idiopathic detrusor instability) PROTEIN SEQUENCE Full mgqagck...lephrst (1..422; 422 aa, pI: 7.63, MW:  
47206 TM: 1 [P] Gene Chromosome: 17p13.3, Genbank accession No. NP\_002552.2)

- Le et al (1997) FEBS Lett. 418(1-2):195-199; WO2004047749; WO2003072035 (claim 10); Touchman et al  
25 (2000) Genome Res. 10:165-173; WO200222660 (claim 20); WO2003093444 (claim 1); WO2003087768  
(claim 1); WO2003029277 (page 82);

(32) CD72 (B-cell differentiation antigen CD72, Lyb-2) PROTEIN SEQUENCE Full maeaity...tafrfpd (1..359;  
359 aa, pI: 8.66, MW: 40225 TM: 1 [P] Gene Chromosome: 9p13.3, Genbank accession No. NP\_001773.1)

- 30 WO2004042346 (claim 65); WO2003026493 (pages 51-52, 57-58); WO200075655 (pages 105-106); Von  
Hoegen et al (1990) J. Immunol. 144(12):4870-4877; Strausberg et al (2002) Proc. Natl. Acad. Sci USA  
99:16899-16903;

(33) LY64 (Lymphocyte antigen 64 (RP105), type I membrane protein of the leucine rich repeat (LRR) family,  
35 regulates B-cell activation and apoptosis, loss of function is associated with increased disease activity in patients  
with systemic lupus erythematosus) PROTEIN SEQUENCE Full mafdvsc...rwkyqhi (1..661; 661 aa, pI: 6.20,  
MW: 74147 TM: 1 [P] Gene Chromosome: 5q12, Genbank accession No. NP\_005573.1)

- US2002193567; WO9707198 (claim 11, pages 39-42); Miura et al (1996) Genomics 38(3):299-304; Miura et al  
(1998) Blood 92:2815-2822; WO2003083047; WO9744452 (claim 8, pages 57-61); WO200012130 (pages 24-  
40 26);

(34) FcRH1 (Fc receptor-like protein 1, a putative receptor for the immunoglobulin Fc domain that contains C2 type Ig-like and ITAM domains, may have a role in B-lymphocyte differentiation) PROTEIN SEQUENCE Full mlprll...vdyedam (1..429; 429 aa), pI: 5.28, MW: 46925 TM: 1 [P] Gene Chromosome: 1q21-1q22, Genbank  
5 accession No. NP\_443170.1)

WO2003077836; WO200138490 (claim 6, Fig 18E-1-18-E-2); Davis et al (2001) Proc. Natl. Acad. Sci USA 98(17):9772-9777; WO2003089624 (claim 8); EP1347046 (claim 1); WO2003089624 (claim 7);

(35) IRTA2 (Immunoglobulin superfamily receptor translocation associated 2, a putative immunoreceptor with  
10 possible roles in B cell development and lymphomagenesis; deregulation of the gene by translocation occurs in some B cell malignancies) PROTEIN SEQUENCE Full mllwvil...assaphr (1..977; 977 aa), pI: 6.88 MW: 106468 TM: 1 [P] Gene Chromosome: 1q21, Genbank accession No. Human:AF343662, AF343663, AF343664, AF343665, AF369794, AF397453, AK090423, AK090475, AL834187, AY358085; Mouse:AK089756, AY158090, AY506558; NP\_112571.1

15 WO2003024392 (claim 2, Fig 97); Nakayama et al (2000) Biochem. Biophys. Res. Commun. 277(1):124-127; WO2003077836; WO200138490 (claim 3, Fig 18B-1-18B-2);

(36) TENB2 (TMEFF2, tomoregulin, TPEF, HPP1, TR, putative transmembrane proteoglycan, related to the EGF/heregulin family of growth factors and follistatin) PROTEIN SEQUENCE Full mvlwesp...rastli (1..374;  
20 374 aa, NCBI Accession: AAD55776, AAF91397, AAG49451, NCBI RefSeq: NP\_057276; NCBI Gene: 23671; OMIM: 605734; SwissProt Q9UIK5; Genbank accession No. AF179274; AY358907, CAF85723, CQ782436

WO2004074320 (SEQ ID NO 810); JP2004113151 (SEQ ID NOS 2, 4, 8); WO2003042661 (SEQ ID NO 580); WO2003009814 (SEQ ID NO 411); EP1295944 (pages 69-70); WO200230268 (page 329); WO200190304  
25 (SEQ ID NO 2706); US2004249130; US2004022727; WO2004063355; US2004197325; US2003232350; US2004005563; US2003124579; US 6410506; US 6642006l; Horie et al (2000) Genomics 67:146-152; Uchida et al (1999) Biochem. Biophys. Res. Commun. 266:593-602; Liang et al (2000) Cancer Res. 60:4907-12; Glynne-Jones et al (2001) Int J Cancer. Oct 15;94(2):178-84.

## PRODUCTION OF ANTIBODIES

30 Various methods have been employed to produce monoclonal antibodies (MAbs). Hybridoma technology, which refers to a cloned cell line that produces a single type of antibody, uses the cells of various species, including mice (murine), hamsters, rats, and humans. Other methods to prepare MAbs, including chimeric and humanized antibodies, uses genetic engineering, i.e. recombinant DNA techniques.

Polyclonal antibodies may be raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip)  
35 injections of the relevant antigen and an adjuvant. Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts.

Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, (1984) J. Immunol., 133:3001, and Brodeur et al.,  
40 *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York,

1987)). Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Binding specificity of monoclonal antibodies produced by hybridoma cells may be determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). The binding affinity of the monoclonal antibody can; for example, be determined by the Scatchard analysis of Munson et al (1980) Anal. Biochem. 107:220.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells (US 2005/0048572; US 2004/0229310). Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al (1993) Curr. Opinion in Immunol. 5:256-262 and Plückthun (1992) Immunol. Revs. 130:151-188.

In a further embodiment, monoclonal antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al (1990) Nature 348:552-554; Clackson et al (1991) Nature 352:624-628; and Marks et al (1991) J. Mol. Biol., 222:581-597 describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al (1992) Bio/Technology 10:779-783), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse et al (1993) Nuc. Acids. Res. 21:2265-2266). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy chain and light chain constant domains in place of the homologous murine sequences (US 4816567); and Morrison et al (1984) Proc. Natl Acad. Sci. USA 81:6851), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

A description follows as to exemplary techniques for the production of the antibodies (Ab) used in the antibody-drug conjugates (ADC) of the present invention. The production of antibodies will be illustrated with reference to anti-ErbB2 antibodies but it will be apparent for those skilled in the art that antibodies to other members of the ErbB receptor family, as well as any other receptor or tumor-associated antigen or target, can be produced and modified in a similar manner.

The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface, e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma

cell line such as SK-BR-3 cells (Stancovski et al (1991) PNAS (USA) 88:8691-8695), can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

Example 1 describes production of an exemplary humanized anti-ErbB2 antibody. The humanized antibody may, for example, comprise nonhuman hypervariable region residues incorporated into a human variable heavy domain and may further comprise a framework region (FR) substitution at a position selected from the group consisting of 69H, 71H and 73H utilizing the variable domain numbering system set forth in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991). In one embodiment, the humanized antibody comprises FR substitutions at two or all of positions 69H, 71H and 73H.

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production (Jakobovits et al (1993) Proc. Natl. Acad. Sci. USA, 90:2551; Jakobovits et al (1993) Nature 362:255-258; Bruggermann et al (1993) Year in Immuno. 7:33; and US 5591669; US 5589369; US 5545807).

Alternatively, phage display technology (McCafferty et al (1990) Nature 348:552-553) can be used to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors (Johnson, Kevin S. and Chiswell, David J. (1993) *Current Opinion in Structural Biology* 3:564-571). A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially (Marks et al (1991) J. Mol. Biol. 222:581-597; Griffith et al (1993) EMBO J. 12:725-734; US 5565332; US 5573905). Human antibodies may also be generated by *in vitro* activated B cells (US 5567610; US 5229275). Human anti-ErbB2 antibodies are described (US 5772997 and WO 97/00271).

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see Morimoto et al (1992) Journal of Biochemical and Biophysical Methods 24:107-117; and Brennan et al (1985) Science 229:81). Antibody fragments can also be produced directly by recombinant host cells and the antibody phage libraries discussed above. Fab'-SH fragments can be directly recovered from E. coli and chemically coupled to form F(ab')<sub>2</sub> fragments (Carter et al (1992) Bio/Technology 10:163-167). According to another approach, F(ab')<sub>2</sub> fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; US 5571894; and US 5587458. The antibody fragment may also be a "linear antibody", *e.g.*, as described in US 5641870 for example. Such linear antibody fragments may be monospecific or bispecific.

Bispecific antibodies with binding specificities for at least two different epitopes (Millstein et al (1983), Nature 305:537-539) may bind to two different epitopes of the ErbB2 protein. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.* CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2 (WO 96/16673; US

5837234; WO98/02463; US 5821337). Purification methods for bispecific antibodies have been disclosed (WO 93/08829; Traunecker et al (1991) EMBO J. 10:3655-3659; WO 94/04690; Suresh et al (1986) *Methods in Enzymology* 121:210; US 5731168). Bispecific antibodies can be produced using leucine zippers (Kostelny et al (1992) J. Immunol. 148(5):1547-1553), and single-chain Fv (sFv) dimers (Gruber et al (1994) J. Immunol. 152:5368).

Techniques for generating bispecific antibodies from antibody fragments have also been described, such as using chemical linkage wherein intact antibodies are proteolytically cleaved to generate F(ab')<sub>2</sub> fragments (Brennan et al (1985) Science 229:81). Fab'-SH fragments can be recovered from *E. coli* and chemically coupled to form bispecific antibodies (Shalaby et al (1992) J. Exp. Med. 175:217-225). The "diabody" technology provides an alternative method for making bispecific antibody fragments (Hollinger et al (1993) Proc. Natl. Acad. Sci. USA 90:6444-6448).

Antibodies with more than two valencies are contemplated. Multivalent, "Octopus" antibodies with three or more antigen binding sites and two or more variable domains can be readily produced by recombinant expression of nucleic acid encoding the polypeptide chains of the antibody (US 2002/0004586; WO 01/77342). For example, trispecific antibodies can be prepared (Tutt et al (1991) J. Immunol. 147:60).

Amino acid sequence modification(s) of antibodies are contemplated. For example, mutants and various isoforms of antibodies which bind to tumor-associated antigens are contemplated to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of an antibody are prepared by introducing appropriate nucleotide changes into the nucleic acid encoding the antibody, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the antibody that are preferred locations for mutagenesis is "alanine scanning mutagenesis" (Cunningham and Wells (1989) Science 244:1081-1085) where an amino acid residue, or group of target residues, are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid, such as alanine or polyalanine, to optimize the interaction of the amino acids with antigen. Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an anti-ErbB2 antibody with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the anti-ErbB2 antibody molecule include the fusion to the N- or C-terminus of the anti-ErbB2 antibody to an enzyme (e.g. for ADEPT: Tietze et al (2003) Current Pharm. Design 9:2155-2175) or a polypeptide which increases the serum half-life of the antibody, such as an albumin-binding peptide.

Plasma-protein binding can be an effective means of improving the pharmacokinetic properties of short lived molecules. Albumin is the most abundant protein in plasma. Serum albumin binding peptides (ABP) can alter the pharmacodynamics of fused active domain proteins, including alteration of tissue uptake, penetration, and diffusion. These pharmacodynamic parameters can be modulated by specific selection of the appropriate

serum albumin binding peptide sequence (US 20040001827). A series of albumin binding peptides were identified by phage display screening (Dennis et al (2002) "Albumin Binding As A General Strategy For Improving The Pharmacokinetics Of Proteins" J Biol Chem. 277:35035-35043; WO 01/45746). Compounds of the invention include ABP sequences taught by: (i) Dennis et al. (2002) J Biol Chem. 277:35035-35043 at Tables III and IV, page 35038; (ii) US 20040001827 at [0076] SEQ ID NOS: 9-22; and (iii) WO 01/45746 at pages 12-13, SEQ ID NOS: z1-z14, and all of which are incorporated herein by reference.

The amino acid sequence is usually altered by altering the underlying nucleic acid sequence. Nucleic acid molecules encoding amino acid sequence variants of the antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated.

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophilic: cys, ser, thr;
- (3) acidic: asp, glu;
- (4) basic: asn, gln, his, lys, arg;
- (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in US 5739277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, or IgG<sub>4</sub>) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule (US 2003/0190311, US6821505; US 6165745; US 5624821; US 5648260; US 6165745; US 5834597).

Glycosylation variants of antibodies are variants in which the glycosylation pattern of an antibody is altered. By altering is meant deleting one or more carbohydrate moieties found in the antibody, adding one or more carbohydrate moieties to the antibody, changing the composition of glycosylation (glycosylation pattern), or the extent of glycosylation.

Antibodies may be glycosylated at conserved positions (N-linked or O-linked) in their constant regions (Hse et al (1997) J. Biol. Chem. 272:9062-9070; Jefferis and Lund, (1997) Chem. Immunol. 65:111-128; Wright and Morrison, (1997) TibTECH 15:26-32). The oligosaccharide side chains of the immunoglobulins affect the protein's function (Boyd et al (1996) Mol. Immunol. 32:1311-1318; Wittwe and Howard, (1990) Biochem. 29:4175-4180), and the intramolecular interaction between portions of the glycoprotein which can affect the conformation and presented three-dimensional surface of the glycoprotein (Hefferis and Lund, *supra*; Wyss and Wagner (1996) Current Opin. Biotech. 7:409-416). Oligosaccharides may also serve to target a given glycoprotein to certain molecules based upon specific recognition structures (Malhotra et al (1995) Nature Med. 1:237-243; Umana et al (1999) Nature Biotech. 17:176-180). Removal of the oligosaccharides may optimize antigen binding and other properties of the antibody (Boyd et al (1996) Mol. Immunol. 32:1311-1318).

Factors which affect glycosylation during recombinant production of antibodies include growth mode, media formulation, culture density, oxygenation, pH, purification schemes and the like (US 5047335; US 5510261; US 5278299). Glycosylation, or certain types of glycosylation, can be enzymatically removed from the glycoprotein, for example using endoglycosidase H (Endo H). In addition, the recombinant host cell can be genetically engineered, e.g. make defective in processing certain types of polysaccharides. These and similar techniques are well known in the art.

The glycosylation structure of antibodies can be readily analyzed by conventional techniques of carbohydrate analysis, including lectin chromatography, NMR, Mass spectrometry, HPLC, GPC, monosaccharide compositional analysis, sequential enzymatic digestion, and HPAEC-PAD, which uses high pH anion exchange chromatography to separate oligosaccharides based on charge. Methods for releasing oligosaccharides for analytical purposes are also known, and include, without limitation, enzymatic treatment (commonly performed using peptide-N-glycosidase F/endo- $\beta$ -galactosidase), elimination using harsh alkaline environment to release mainly O-linked structures, and chemical methods using anhydrous hydrazine to release both N- and O-linked oligosaccharides.

#### MAYTANSINOID DRUG MOIETIES

Maytansine compounds inhibit cell proliferation by inhibiting the formation of microtubules during mitosis through inhibition of polymerization of the microtubulin protein, tubulin (Remillard et al (1975) Science 189:1002-1005; US 5208020). Maytansine and maytansinoids are highly cytotoxic but their clinical use in cancer therapy has been greatly limited by their severe systemic side-effects primarily attributed to their poor selectivity for tumors. Clinical trials with maytansine had been discontinued due to serious adverse effects on the central nervous system and gastrointestinal system (Issel et al (1978) Can. Treatment. Rev. 5:199-207).

Maytansinoid drug moieties are attractive drug moieties in antibody-drug conjugates because they are: (i) relatively accessible to prepare by fermentation or chemical modification, derivatization of fermentation products, (ii) amenable to derivatization with functional groups suitable for conjugation through the non-disulfide linkers to antibodies, (iii) stable in plasma, and (iv) effective against a variety of tumor cell lines.

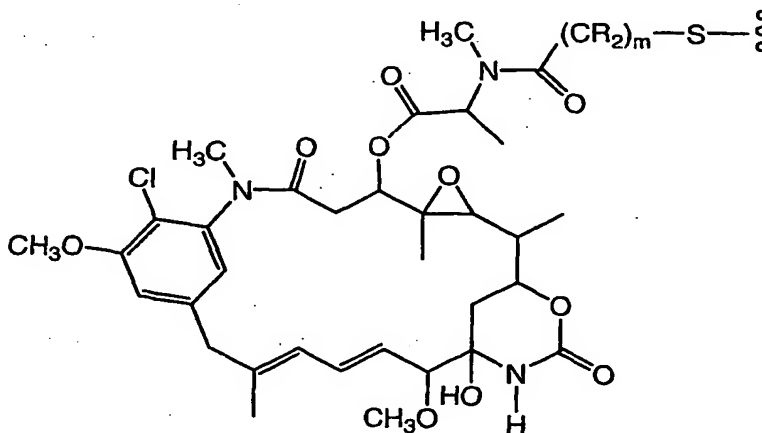
Maytansine compounds suitable for use as maytansinoid drug moieties are well known in the art, and can be isolated from natural sources according to known methods, produced using genetic engineering techniques (see Yu et al (2002) PNAS 99:7968-7973), or maytansinol and maytansinol analogues prepared synthetically according to known methods.

Exemplary maytansinoid drug moieties include those having a modified aromatic ring, such as: C-19-dechloro (US 4256746) (prepared by lithium aluminum hydride reduction of ansamitocin P2); C-20-hydroxy (or C-20-demethyl) +/-C-19-dechloro (US Pat. Nos. 4361650 and 4307016) (prepared by demethylation using *Streptomyces* or *Actinomyces* or dechlorination using LAH); and C-20-demethoxy, C-20-acyloxy (-OCOR), +/-dechloro (US 4294757) (prepared by acylation using acyl chlorides). and those having modifications at other positions

Exemplary maytansinoid drug moieties also include those having modifications such as: C-9-SH, prepared by the reaction of maytansinol with H<sub>2</sub>S or P<sub>2</sub>S<sub>5</sub> (US 4424219); C-14-alkoxymethyl(demethoxy/CH<sub>2</sub>OR)(US 4331598); C-14-hydroxymethyl or acyloxymethyl (CH<sub>2</sub>OH or CH<sub>2</sub>OAc) prepared from *Nocardia* (US 4450254); C-15-hydroxy/acyloxy, prepared by the conversion of maytansinol by *Streptomyces* (US 4364866); C-15-methoxy, isolated from *Trewia nudiflora* (US 4313946 and US 4315929); C-18-N-demethyl, prepared by the demethylation of maytansinol by *Streptomyces* (US 4362663 and US 4322348); and 4,5-deoxy, prepared by the titanium trichloride/LAH reduction of maytansinol (US 4371533).

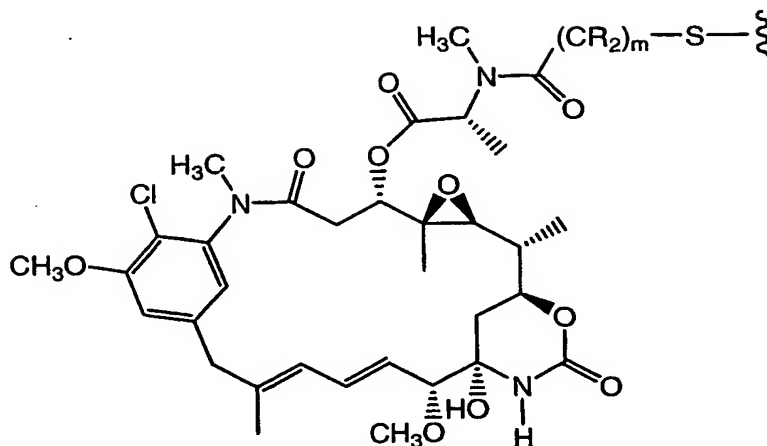
Many positions on maytansine compounds are known to be useful as the linkage position, depending upon the type of link. For example, for forming an ester linkage, the C-3 position having a hydroxyl group, the C-14 position modified with hydroxymethyl, the C-15 position modified with a hydroxyl group and the C-20 position having a hydroxyl group are all suitable.

Maytansinoid drug moieties (D) include those having the structure:



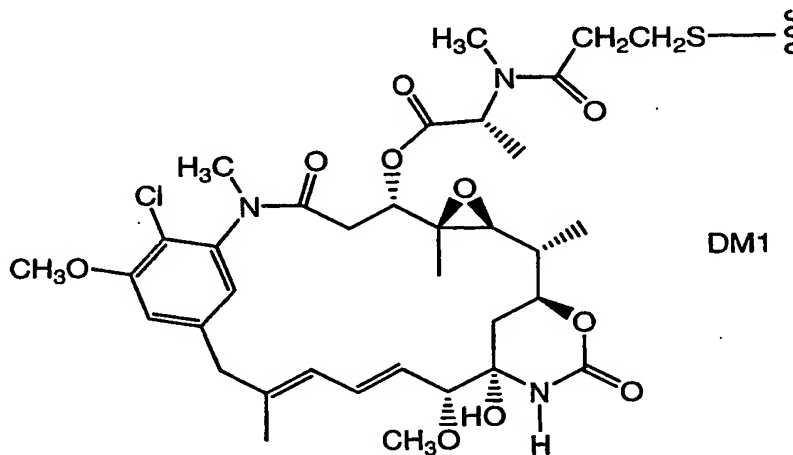
where the wavy line indicates the covalent attachment of the sulfur atom of D to a linker (L) of an antibody-drug conjugate (ADC). R may independently be H or a C<sub>1</sub>-C<sub>6</sub> alkyl. The alkylene chain attaching the amide group to the sulfur atom may be methanyl, ethanyl, or propyl, i.e. m is 1, 2, or 3 (US 633410, 5208020, Chari et al (1992) Cancer Res. 52:127-131; Liu et al (1996) Proc. Natl. Acad. Sci 93:8618-8623).

All stereoisomers of the maytansinoid drug moiety are contemplated for the compounds of the invention, i.e. any combination of R and S configurations at the chiral carbons of D. In one embodiment, the maytansinoid drug moiety (D) will have the following stereochemistry:



Embodiments of D include:

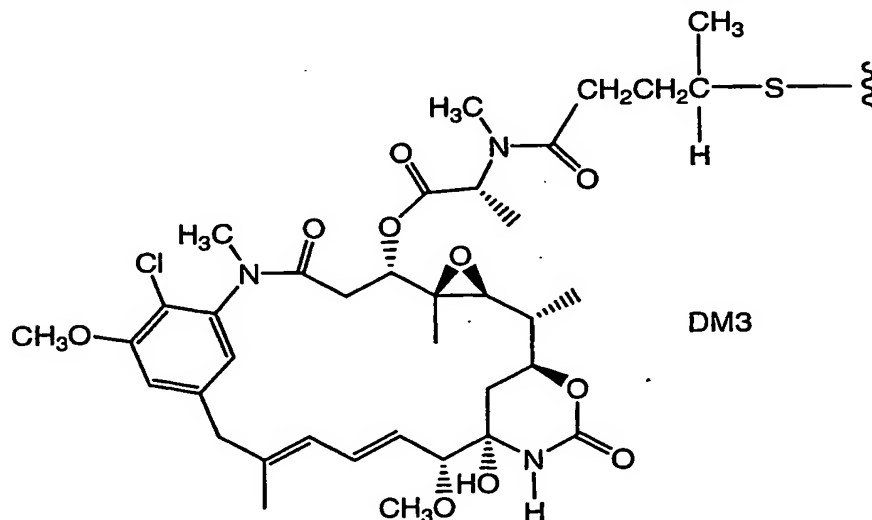
DM1 ( $N^{2'}$ -deacetyl- $N^{2'}$ -(3-mercapto-1-oxopropyl)-maytansine) where  $(CR_2)_m = CH_2CH_2$ ;



DM1

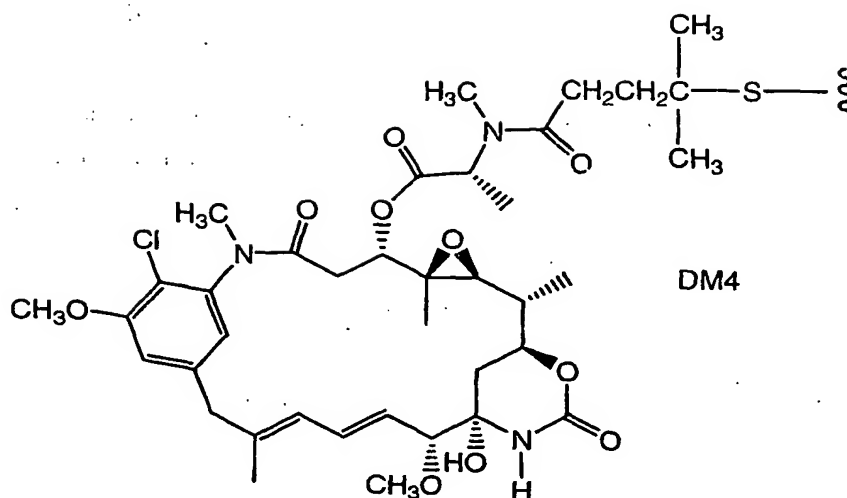
5

DM3 ( $N^{2'}$ -deacetyl- $N^{2'}$ -(4-mercapto-1-oxopentyl)-maytansine) where  $(CR_2)_m = CH_2CH_2CH(CH_3)$ ;



DM3

DM4 ( $N^{2'}$ -deacetyl- $N^{2'}$ -(4-methyl-4-mercapto-1-oxopentyl)-maytansine) where  $(CR_2)_m =$



Steric hindrance conferred by alkyl groups such as the methyl groups on the carbon adjacent to the sulfur atom of DM3 and DM4 may affect the rate of intracellular cleavage of the ADC (US 2004/0235840 A1).

- 5 The variable alkyl unit (CR<sub>2</sub>)<sub>m</sub> may therefore affect potency, efficacy, and safety/toxicity in vitro and in vivo.

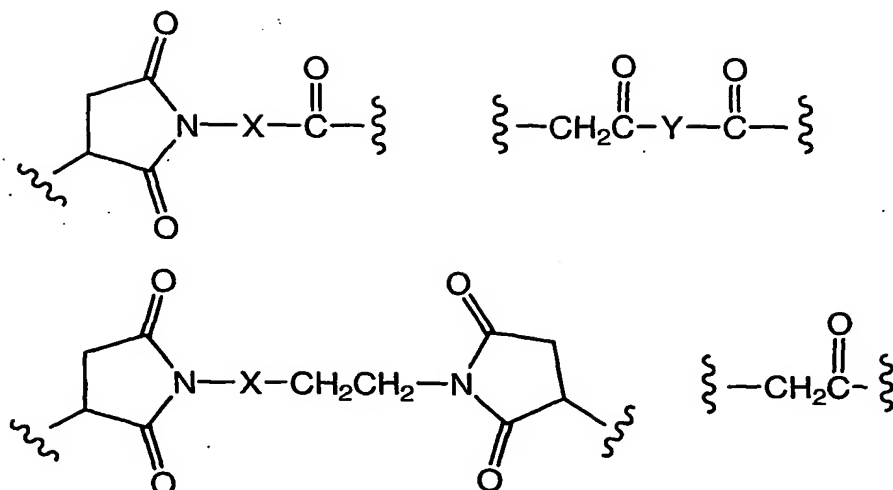
#### LINKERS

The linker, L, attaches the antibody to a drug moiety through covalent bond(s), not comprising a disulfide group. The linker is a bifunctional or multifunctional moiety which can be used to link one or more Drug moieties (D) and an antibody unit (Ab) to form antibody-drug conjugates (ADC) of Formula I. Antibody-drug conjugates (ADC) can be conveniently prepared using a linker having reactive functionality for binding to the Drug and to the Antibody. A cysteine thiol, or an amine, e.g. N-terminus or amino acid side chain such as lysine, of the antibody (Ab) can form a bond with a functional group of a linker reagent, drug moiety or drug-linker reagent.

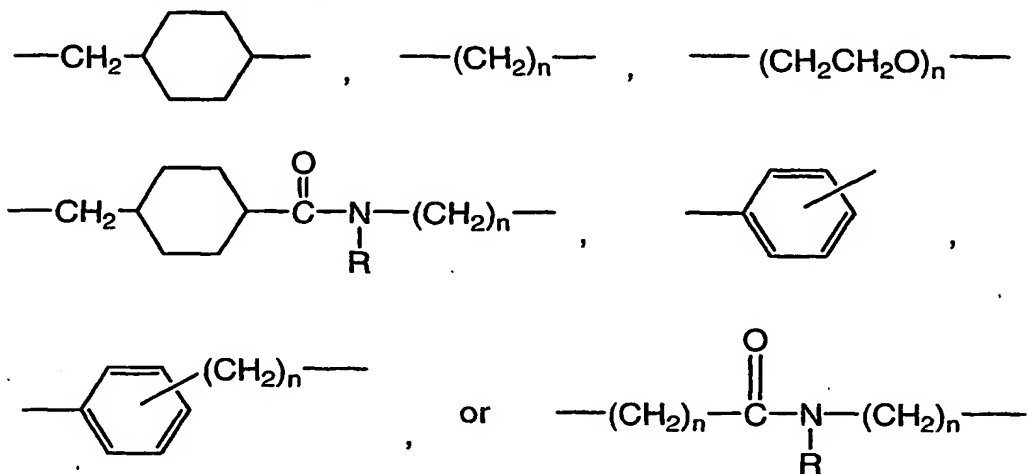
15 The linkers are preferably stable extracellularly. Before transport or delivery into a cell, the antibody-drug conjugate (ADC) is preferably stable and remains intact, i.e. the antibody remains linked to the drug moiety. The linkers are stable outside the target cell and may be cleaved at some efficacious rate inside the cell. An effective linker will: (i) maintain the specific binding properties of the antibody; (ii) allow intracellular delivery of the conjugate or drug moiety; (iii) remain stable and intact, i.e. not cleaved, until the conjugate has been delivered or transported to its targetted site; and (iv) maintain a cytotoxic, cell-killing effect or a cytostatic effect of the maytansinoid drug moiety. Stability of the ADC may be measured by standard analytical techniques such as mass spectroscopy, HPLC, and the separation/analysis technique LC/MS.

20 Covalent attachment of the antibody and the drug moiety requires the linker to have two reactive functional groups, i.e. bivalency in a reactive sense. Bivalent linker reagents which are useful to attach two or more functional or biologically active moieties, such as peptides, nucleic acids, drugs, toxins, antibodies, haptens, and reporter groups are known, and methods have been described their resulting conjugates (Hermanson, G.T. (1996) *Bioconjugate Techniques*; Academic Press: New York, p234-242).

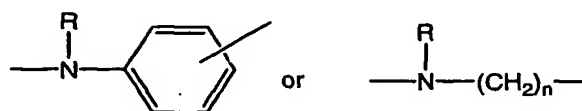
Linkers may have structures selected from:



where the wavy lines indicate the covalent attachments to Ab and D in either orientation. X may have the structures, in either orientation:

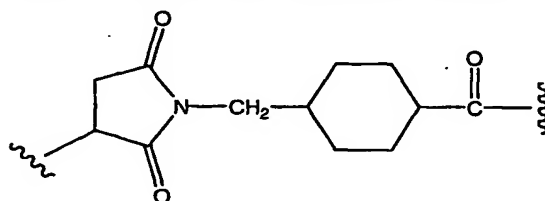


5 where R is independently H or C<sub>1</sub>-C<sub>6</sub> alkyl; and n is 1 to 12. Y may have the structures, in either orientation:

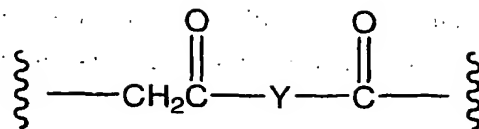


where R is independently H or C<sub>1</sub>-C<sub>6</sub> alkyl; and n is 1 to 12.

For example, the linker may have the structure, designated as SMCC:

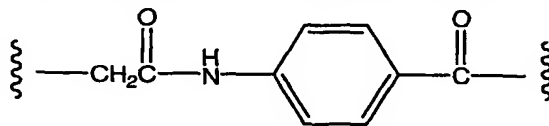


10 In another embodiment, linker (L) has the structure:



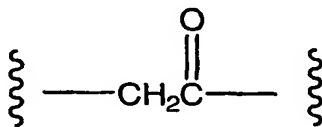
where the wavy lines indicate the covalent attachments to Ab and D in either orientation.

For example, the linker may have the structure, designated as SIAB:



5

In another embodiment, linker (L) has the structure:



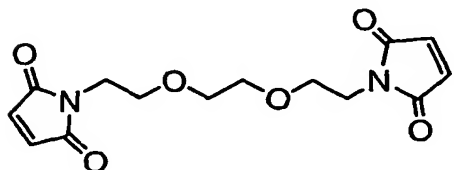
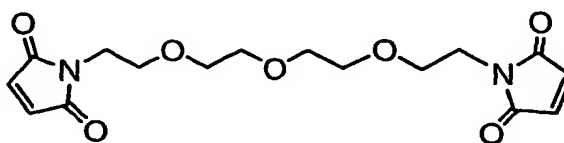
10 In another embodiment, the linker may be substituted with groups which modulated solubility or reactivity. For example, a sulfonate substituent may increase water solubility of the reagent and facilitate the coupling reaction of the linker reagent with the antibody or the drug moiety, or facilitate the coupling reaction of Ab-L with D, or D-L with Ab, depending on the synthetic route employed to prepare the ADC.

15 In another embodiment, a Linker has a reactive functional group which has a nucleophilic group that is reactive to an electrophilic group present on an antibody. Useful electrophilic groups on an antibody include, but are not limited to, aldehyde and ketone carbonyl groups. The heteroatom of a nucleophilic group of a Linker can react with an electrophilic group on an antibody and form a covalent bond to an antibody unit. Useful nucleophilic groups on a Linker include, but are not limited to, hydrazide, oxime, amino, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide. The electrophilic group on an antibody provides  
20 a convenient site for attachment to a Linker.

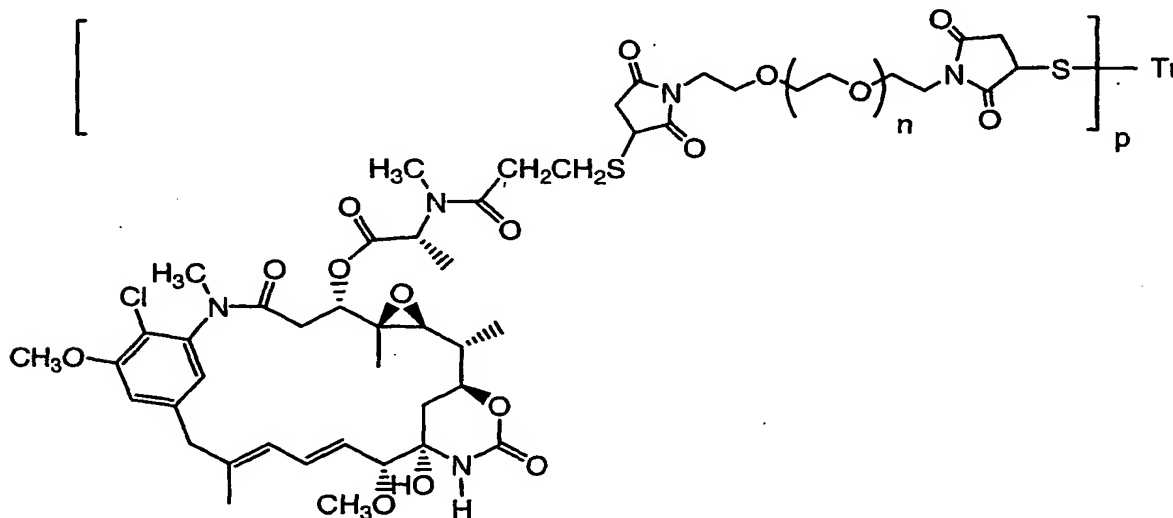
Linkers can be peptidic, comprising one or more amino acid units. Peptide linker reagents may be prepared by solid phase or liquid phase synthesis methods (E. Schröder and K. Lübke, *The Peptides*, volume 1, pp 76-136 (1965) Academic Press) that are well known in the field of peptide chemistry, including t-BOC chemistry (Geiser et al "Automation of solid-phase peptide synthesis" in *Macromolecular Sequencing and*  
25 *Synthesis*, Alan R. Liss, Inc., 1988, pp. 199-218) and Fmoc/HBTU chemistry (Fields, G. and Noble, R. (1990) "Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids", *Int. J. Peptide Protein Res.* 35:161-214), on an automated synthesizer such as the Rainin Symphony Peptide Synthesizer (Protein Technologies, Inc., Tucson, AZ), or Model 433 (Applied Biosystems, Foster City, CA).

30 The compounds expressly contemplate, but are not limited to, ADC prepared with cross-linker reagents: BMPEO, BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate), and including bis-maleimide reagents: DTME,

BMB, BMDB, BMH, BMOE, BM(PEO)<sub>3</sub>, and BM(PEO)<sub>4</sub>, which are commercially available from Pierce Biotechnology, Inc., Customer Service Department, P.O. Box 117, Rockford, IL. 61105 U.S.A, U.S.A 1-800-874-3723, International +815-968-0747. See pages 467-498, 2003-2004 Applications Handbook and Catalog. Bis-maleimide reagents allow the attachment of a free thiol group of a cysteine residue of an antibody to a thiol-containing drug moiety, label, or linker intermediate, in a sequential or concurrent fashion. Other functional groups besides maleimide, which are reactive with a thiol group of an antibody, maytansinoid drug moiety, or linker intermediate include iodoacetamide, bromoacetamide, vinyl pyridine, disulfide, pyridyl disulfide, isocyanate, and isothiocyanate.

BM(PEO)<sub>3</sub>BM(PEO)<sub>4</sub>

- 10 Exemplary antibody-drug conjugates where DM1 is linked through a BMPEO linker to a thiol group of trastuzumab have the structure:



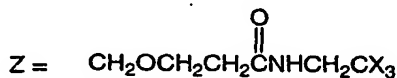
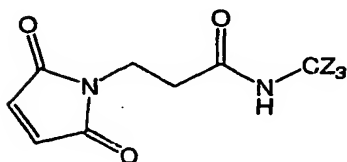
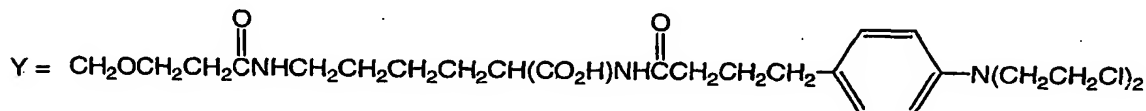
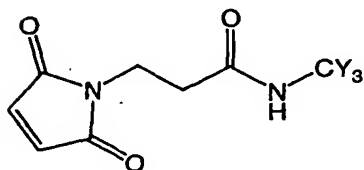
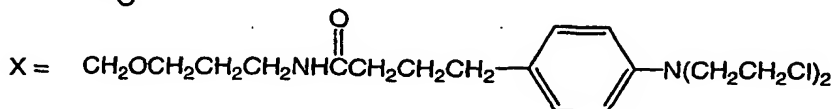
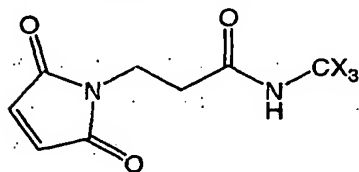
where Tr is trastuzumab; n is 0, 1, or 2; and p is 1, 2, 3, or 4.

Useful linker reagents can also be obtained via other commercial sources, such as Molecular

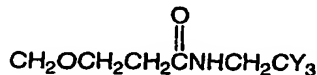
- 15 Biosciences Inc.(Boulder, CO), or synthesized in accordance with procedures described in Toki et al (2002) J. Org. Chem. 67:1866-1872; US 6214345 to Firestone et al; WO 02/088172; US 2003130189; US2003096743; WO 03/026577; WO 03/043583; and WO 04/032828.

- The Linker may be a dendritic type linker for covalent attachment of more than one drug moiety through a branching, multifunctional linker moiety to an antibody (Sun et al (2002) Bioorganic & Medicinal Chemistry Letters 12:2213-2215; Sun et al (2003) Bioorganic & Medicinal Chemistry 11:1761-1768; King et al (2002) Tetrahedron Letters 43:1987-1990). Dendritic linkers can increase the molar ratio of drug to antibody, i.e. loading, which is related to the potency of the ADC. Thus, where an antibody bears only one reactive cysteine thiol group, a multitude of drug moieties may be attached through a dendritic linker.
- 20

The following exemplary embodiments of dendritic linker reagents allow up to nine nucleophilic drug moiety reagents to be conjugated by reaction with the chloroethyl nitrogen mustard functional groups:



or



## DRUG LOADING

The drug loading is represented by p in a molecule of Formula I, the average number of maytansinoid drugs per antibody. Drug loading may range from 1 to 8 drugs (D) per antibody (Ab), i.e. where 1, 2, 3, 4, 5, 6, 7, and 8 drug moieties are covalently attached to the antibody. Compositions of ADC of Formula I include collections of antibodies conjugated with a range of drugs, from 1 to 8. The average number of drugs per antibody in preparations of ADC from conjugation reactions may be characterized by conventional means such as mass spectroscopy, ELISA assay, electrophoresis, and HPLC. The quantitative distribution of ADC in terms of p may also be determined. By ELISA, the averaged value of p in a particular preparation of ADC may be determined (Hamblett et al (2004) Clinical Cancer Res. 10:7063-7070; Sanderson et al (2005) Clinical Cancer Res. 11:843-852). However, the distribution of p (drug) values is not discernible by the antibody-antigen binding and detection limitation of ELISA. Also, ELISA assay for detection of antibody-drug conjugates does not determine where the drug moieties are attached to the antibody, such as the heavy chain or light chain fragments, or the particular amino acid residues. In some instances, separation, purification, and

characterization of homogeneous ADC where  $p$  is a certain value from ADC with other drug loadings may be achieved by means such as reverse phase HPLC or electrophoresis.

For some antibody-drug conjugates,  $p$  may be limited by the number of attachment sites on the antibody. For example, where the attachment is a cysteine thiol, as in the exemplary embodiments above, an antibody may have only one or several cysteine thiol groups, or may have only one or several sufficiently reactive thiol groups through which a linker may be attached. Higher drug loading, e.g.  $p > 5$ , may cause aggregation, insolubility, toxicity, or loss of cellular permeability of certain antibody-drug conjugates.

Typically, fewer than the theoretical maximum of drug moieties are conjugated to an antibody during a conjugation reaction. An antibody may contain, for example, many lysine residues that do not react with the drug-linker intermediate (D-L) or linker reagent. Only the most reactive lysine groups may react with an amine-reactive linker reagent. Also, only the most reactive cysteine thiol groups may react with a thiol-reactive linker reagent. Generally, antibodies do not contain many, if any, free and reactive cysteine thiol groups which may be linked to a drug moiety. Most cysteine thiol residues in the antibodies of the compounds exist as disulfide bridges and must be reduced with a reducing agent such as dithiothreitol (DTT) or TCEP, under partial or total reducing conditions. Additionally, the antibody must be subjected to denaturing conditions to reveal reactive nucleophilic groups such as lysine or cysteine. The loading (drug/antibody ratio) of an ADC may be controlled in several different manners, including: (i) limiting the molar excess of drug-linker intermediate (D-L) or linker reagent relative to antibody, (ii) limiting the conjugation reaction time or temperature, and (iii) partial or limiting reductive conditions for cysteine thiol modification.

Where more than one nucleophilic or electrophilic group of the antibody reacts with a drug-linker intermediate, or linker reagent followed by drug moiety reagent, then the resulting product is a mixture of ADC compounds with a distribution of drug moieties attached to an antibody, e.g. 1, 2, 3, etc. Liquid chromatography methods such as polymeric reverse phase (PLRP) and hydrophobic interaction (HIC) may separate compounds in the mixture by drug loading value. Preparations of ADC with a single drug loading value ( $p$ ) may be isolated ("Effect of drug loading on the pharmacology, pharmacokinetics, and toxicity of an anti-CD30 antibody-drug conjugate", Hamblett, K.J., et al, Abstract No. 624, American Association for Cancer Research; 2004 Annual Meeting, March 27-31, 2004, Proceedings of the AACR, Volume 45, March 2004; "Controlling the Location of Drug Attachment in Antibody-Drug Conjugates", Alley, S.C., et al, Abstract No. 627, American Association for Cancer Research; 2004 Annual Meeting, March 27-31, 2004, Proceedings of the AACR, Volume 45, March 2004). However, these single loading value ADCs may still be heterogeneous mixtures because the drug moieties may be attached, via the linker, at different sites on the antibody.

#### PREPARATION OF ANTIBODY-DRUG CONJUGATES

The ADC of Formula I may be prepared by several routes, employing organic chemistry reactions, conditions, and reagents known to those skilled in the art, including: (1) reaction of a nucleophilic group or an electrophilic group of an antibody with a bivalent linker reagent, to form antibody-linker intermediate Ab-L, via a covalent bond, followed by reaction with an activated drug moiety D; and (2) reaction of a nucleophilic group or an electrophilic group of a drug moiety with a linker reagent, to form drug-linker intermediate D-L, via a covalent bond, followed by reaction with the nucleophilic group or an electrophilic group of an antibody. Conjugation methods (1) and (2) may be employed with a variety of antibodies, drug moieties, and linkers to prepare the antibody-drug conjugates of Formula I.

Nucleophilic groups on antibodies include, but are not limited to: (i) N-terminal amine groups, (ii) side chain amine groups, e.g. lysine, (iii) side chain thiol groups, e.g. cysteine, and (iv) sugar hydroxyl or amino groups where the antibody is glycosylated. Amine, thiol, and hydroxyl groups are nucleophilic and capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups. Certain antibodies have reducible interchain disulfides, i.e. cysteine bridges. Antibodies may be made reactive for conjugation with linker reagents by treatment with a reducing agent such as DTT (Cleland's reagent, dithiothreitol) or TCEP (tris(2-carboxyethyl)phosphine hydrochloride; Getz et al (1999) Anal. Biochem. Vol 273:73-80; Soltec Ventures, Beverly, MA). Each cysteine disulfide bridge will thus form, theoretically, two reactive thiol nucleophiles. Additional nucleophilic groups can be introduced into antibodies through the reaction of lysines with 2-iminothiolane (Traut's reagent) resulting in conversion of an amine into a thiol.

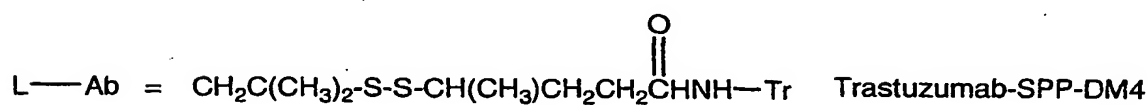
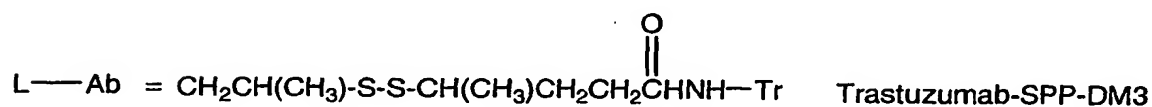
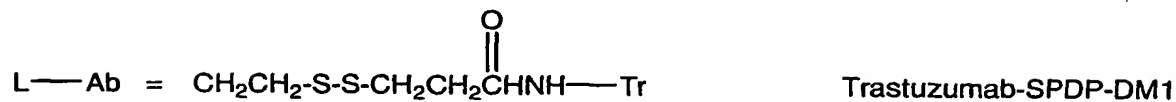
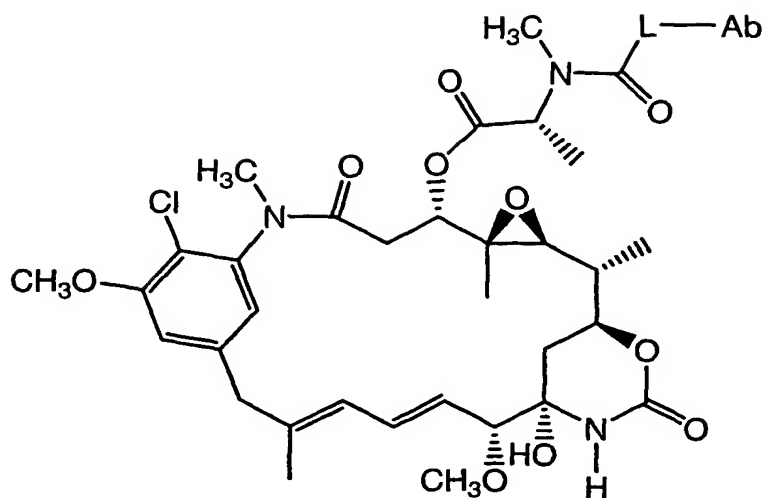
Antibody-drug conjugates may also be produced by modification of the antibody to introduce electrophilic moieties, which can react with nucleophilic substituents on the linker reagent or drug. The sugars of glycosylated antibodies may be oxidized, e.g. with periodate oxidizing reagents, to form aldehyde or ketone groups which may react with the amine group of linker reagents or drug moieties. The resulting imine Schiff base groups may form a stable linkage, or may be reduced, e.g. by borohydride reagents to form stable amine linkages. In one embodiment, reaction of the carbohydrate portion of a glycosylated antibody with either galactose oxidase or sodium meta-periodate may yield carbonyl (aldehyde and ketone) groups in the protein that can react with appropriate groups on the drug (Hermanson, G.T. (1996) *Bioconjugate Techniques*; Academic Press: New York, p234-242). In another embodiment, proteins containing N-terminal serine or threonine residues can react with sodium meta-periodate, resulting in production of an aldehyde in place of the first amino acid (Geoghegan & Stroh, (1992) *Bioconjugate Chem.* 3:138-146; US 5362852). Such aldehyde can be reacted with a drug moiety or linker nucleophile.

Likewise, nucleophilic groups on a drug moiety include, but are not limited to: amine, thiol, hydroxyl, hydrazide, oxime, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide groups capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups.

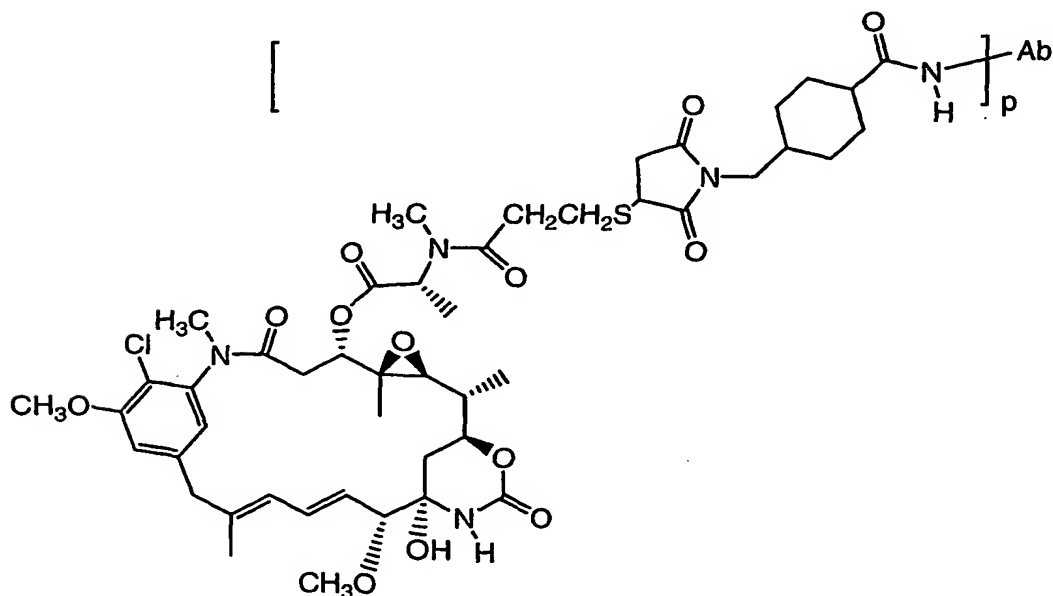
Maytansine may, for example, be converted to May-SSCH<sub>3</sub>, which can be reduced to the free thiol, May-SH, and reacted with a modified antibody (Chari et al (1992) *Cancer Research* 52:127-131) to generate a maytansinoid-antibody immunoconjugate with a disulfide linker. Antibody-maytansinoid conjugates with disulfide linkers have been reported (WO 04/016801; US 6884874; US 2004/039176 A1; WO 03/068144; US 2004/001838 A1; US 6441163; US 5208020; US 5416064; WO 01/024763). The disulfide linker SPP is constructed with linker reagent N-succinimidyl 4-(2-pyridylthio) pentanoate. Antibody-SPP-DM1 conjugates are represented by the structure:

Disulfide (S-S) linker antibody-drug conjugates were tested for purposes of comparison to the non-disulfide linker ADC of the invention. Trastuzumab-SPP-DM1 was prepared according to Example 3 (Ranson, M. and Sliwowski M. (2002) *Oncology* 63(suppl 1):17-24). Disulfide antibody-drug conjugates: trastuzumab-SPDP-DM1, trastuzumab-SPP-DM3, and trastuzumab-SPP-DM4 were also tested, and have the structures

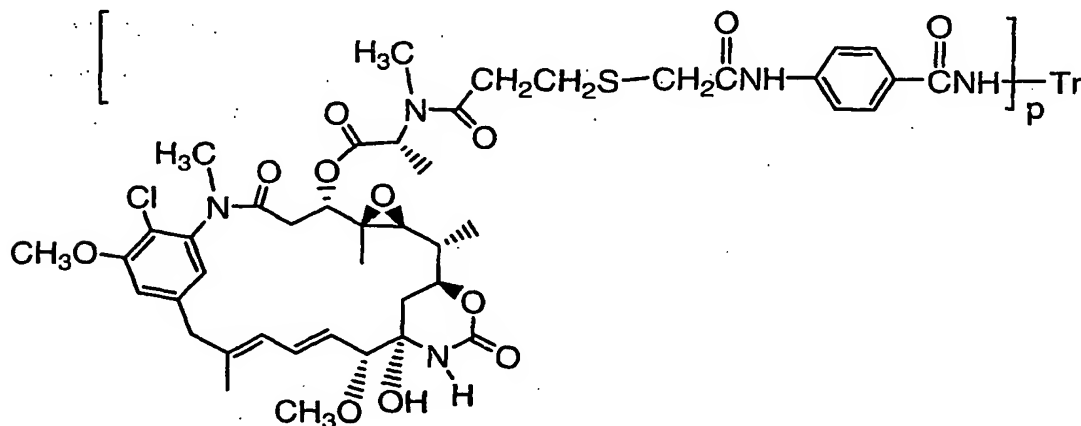
below:



- 5 ADC of the invention include SMCC linkers and the DM1 maytansinoid drug moiety, represented as Ab-SMCC-DM1:



One embodiment of Ab-SMCC-DM1 is trastuzumab-SMCC-DM1 where p is 1, 2, 3, or 4 (Ab = trastuzumab, Tr, WO 2005/037992). Another embodiment of an ADC is trastuzumab-SIAB-DM1 (trastuzumab = Tr) having the structure:



#### SCREENING FOR ANTIBODY-DRUG CONJUGATES (ADC) DIRECTED AGAINST TUMOR-ASSOCIATED ANTIGENS AND CELL SURFACE RECEPTORS

Transgenic animals and cell lines are particularly useful in screening antibody-drug conjugates (ADC) that have potential as prophylactic or therapeutic treatments of diseases or disorders involving overexpression of tumor-associated antigens and cell surface receptors, e.g. HER2 (US 6632979). Screening for a useful ADC may involve administering candidate ADC over a range of doses to the transgenic animal, and assaying at various time points for the effect(s) of the ADC on the disease or disorder being evaluated. Alternatively, or additionally, the drug can be administered prior to or simultaneously with exposure to an inducer of the disease, if applicable. Candidate ADC may be screened serially and individually, or in parallel under medium or high-throughput screening format. The rate at which ADC may be screened for utility for prophylactic or therapeutic treatments of diseases or disorders is limited only by the rate of synthesis or screening methodology, including detecting/measuring/analysis of data.

One embodiment is a screening method comprising (a) transplanting cells from a stable breast cancer cell line into a non-human animal, (b) administering an ADC drug candidate to the non-human animal and (c) determining the ability of the candidate to inhibit the formation of tumors from the transplanted cell line. The invention also concerns a method of screening ADC candidates for the treatment of a disease or disorder characterized by the overexpression of a receptor protein comprising (a) contacting cells from a stable breast cancer cell line with a drug candidate and (b) evaluating the ability of the ADC candidate to inhibit the growth of the stable cell line.

One embodiment is a screening method comprising (a) contacting cells from a stable breast cancer cell line with an ADC drug candidate and (b) evaluating the ability of the ADC candidate to block ligand activation of HER2. Another embodiment the ability of the ADC candidate to block heregulin binding is evaluated. In another embodiment the ability of the ADC candidate to block ligand-stimulated tyrosine phosphorylation is evaluated.

Another embodiment is a screening method comprising (a) contacting cells from a stable breast cancer cell line with an ADC drug candidate and (b) evaluating the ability of the ADC candidate to induce cell death. In one embodiment the ability of the ADC candidate to induce apoptosis is evaluated.

5 Another embodiment is a screening method comprising (a) administering an ADC drug candidate to a transgenic non-human mammal that overexpresses, e.g. in its mammary gland cells, a native human protein, e.g. HER2 or a fragment thereof, wherein such transgenic mammal has stably integrated into its genome a nucleic acid sequence encoding the native human protein or a fragment thereof having the biological activity of the native human protein, operably linked to transcriptional regulatory sequences directing its expression, and develops a tumor, e.g. a mammary tumor, not responding or poorly responding to antibody treatment, e.g. anti-  
10 HER2, or to a non-human mammal bearing a tumor transplanted from said transgenic non-human mammal; and (b) evaluating the effect of the ADC candidate on the target disease or disorder. Without limitations, the disease or disorder may be a HER2-overexpressing cancer, such as breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic and bladder cancer. The cancer may be breast cancer which expressed HER2 in at least about 500,000 copies per cell, or at least about 2,000,000 copies per cell. ADC drug  
15 candidates may, for example, be evaluated for their ability to induce cell death and/or apoptosis, using assay methods well known in the art and described hereinafter.

In one embodiment, candidate ADC are screened by being administered to the transgenic animal over a range of doses, and evaluating the animal's physiological response to the compounds over time. Administration may be oral, or by suitable injection, depending on the chemical nature of the compound being evaluated. In  
20 some cases, it may be appropriate to administer the compound in conjunction with co-factors that would enhance the efficacy of the compound. If cell lines derived from the subject transgenic animals are used to screen for compounds useful in treating various disorders associated with overexpression of certain tumor-associated antigen proteins or cell surface receptors, e.g. HER2-overexpression, the test compounds are added to the cell culture medium at an appropriate time, and the cellular response to the compound is evaluated over time  
25 using the appropriate biochemical and/or histological assays. In some cases, it may be appropriate to apply the compound of interest to the culture medium in conjunction with co-factors that would enhance the efficacy of the compound.

Thus, the present invention provides assays for identifying ADC which specifically target and bind the overexpressed HER2 protein, the presence of which is correlated with abnormal cellular function, and in the  
30 pathogenesis of cellular proliferation and/or differentiation of mammary gland that is causally related to the development of breast tumors.

To identify an ADC which blocks ligand activation of an ErbB (e.g. ErbB2) receptor, the ability of the compound to block ErbB ligand binding to cells expressing the ErbB (ErbB2) receptor (e.g. in conjugation with another ErbB receptor with which the ErbB receptor of interest forms an ErbB hetero-oligomer) may be  
35 determined. For example, cells isolated from the transgenic animal overexpressing HER2 and transfected to express another ErbB receptor (with which HER2 forms hetero-oligomer) may be incubated, i.e. culturing, with the ADC and then exposed to labeled ErbB ligand. The ability of the compound to block ligand binding to the ErbB receptor in the ErbB hetero-oligomer may then be evaluated.

For example, inhibition of heregulin (HRG) binding to breast tumor cell lines, overexpressing HER2  
40 and established from the transgenic non-human mammals (e.g. mice) herein, by the candidate ADC may be

performed using monolayer cultures on ice in a 24-well-plate format. Anti-ErbB2 monoclonal antibodies may be added to each well and incubated for 30 minutes. <sup>125</sup>I-labeled rHRGβ<sub>1177-224</sub> (25,000 cpm) may then be added, and the incubation may be continued for 4 to 16 hours. Dose response curves may be prepared and an IC<sub>50</sub> value may be calculated for the compound of interest.

5 Alternatively, or additionally, the ability of an ADC to block ErbB ligand-stimulated tyrosine phosphorylation of an ErbB receptor present in an ErbB hetero-oligomer may be assessed. For example, cell lines established from the transgenic animals herein may be incubated with a test ADC and then assayed for ErbB ligand-dependent tyrosine phosphorylation activity using an anti-phosphotyrosine monoclonal antibody (which is optionally conjugated with a detectable label). The kinase receptor activation assay described in US  
10 5766863 is also available for determining ErbB receptor activation and blocking of that activity by the compound.

In one embodiment, one may screen for ADC which inhibit HRG stimulation of p180 tyrosine phosphorylation in MCF7 cells essentially as described below. For example, a cell line established from a HER2-transgenic animal may be plated in 24-well plates and the compound may be added to each well and  
15 incubated for 30 minutes at room temperature; then rHRGβ<sub>1177-244</sub> may be added to each well to a final concentration of 0.2 nM, and the incubation may be continued for about 8 minutes. Media may be aspirated from each well, and reactions may be stopped by the addition of 100 μl of SDS sample buffer (5% SDS, 25 mM DTT, and 25 mM Tris-HCl, pH 6.8). Each sample (25 μl) may be electrophoresed on a 4-12% gradient gel (Novex) and then electrophoretically transferred to polyvinylidene difluoride membrane. Antiphosphotyrosine  
20 (at 1 μg/ml) immunoblots may be developed, and the intensity of the predominant reactive band at M<sub>r</sub> -180,000 may be quantified by reflectance densitometry. An alternate method to evaluate inhibition of receptor phosphorylation is the KIRA (kinase receptor activation) assay (Sadick et al (1998) Jour. of Pharm. and Biomed. Anal. 1-9). Some of the well-established monoclonal antibodies against HER2 that are known to inhibit HRG stimulation of p180 tyrosine phosphorylation can be used as positive control in this assay. A dose-response  
25 curve for inhibition of HRG stimulation of p180 tyrosine phosphorylation as determined by reflectance densitometry may be prepared and an IC<sub>50</sub> for the compound of interest may be calculated.

One may also assess the growth inhibitory effects of a test ADC on cell lines derived from a HER2-transgenic animal (Schaefer et al (1997) Oncogene 15:1385-1394). According to this assay, the cells may be treated with a test compound at various concentrations for 4 days and stained with crystal violet or the redox dye  
30 Alamar Blue. Incubation with the compound may show a growth inhibitory effect on this cell line similar to that displayed by monoclonal antibody 2C4 on MDA-MB-175 cells (Schaefer et al., supra). In a further embodiment, exogenous HRG will not significantly reverse this inhibition.

To identify growth inhibitory ADC compounds that specifically target HER2, one may screen for ADC which inhibit the growth of HER2-overexpressing cancer cells derived from transgenic animals (US 5677171).  
35 According to this assay, HER2 overexpressing cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillin streptomycin. The cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35mm dish) and the test compound is added at various concentrations. After six days, the number of cells, compared to untreated cells is counted using an electronic

COULTER™ cell counter. Those ADC which inhibit cell growth by about 20-100% or about 50-100% may be selected as growth inhibitory compounds.

To select for ADC which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake may be assessed relative to control. The PI uptake assay uses cells isolated from the breast tumor tissue of a transgenic animal. According to this assay, the cells are cultured in Dulbecco's Modified Eagle Medium (D-MEM):Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. Thus, the assay is performed in the absence of complement and immune effector cells. The cells are seeded at a density of  $3 \times 10^6$  per dish in 100 x 20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing various concentrations of the compound. The cells are incubated for a 3-day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4 °C, the pellet resuspended in 3 ml cold  $\text{Ca}^{2+}$  binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM  $\text{CaCl}_2$ ) and aliquoted into 35 mm strainer-capped 12 x 75 mm tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 µg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those compounds which induce statistically significant levels of cell death as determined by PI uptake may be selected as cell death-inducing compounds.

In order to select for compounds which induce apoptosis, an annexin binding assay using cells established from the breast tumor tissue of the transgenic animal is performed. The cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 µg/ml of the antibody-drug conjugate (ADC). Following a three-day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in  $\text{Ca}^{2+}$  binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FITC) (1 µg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those compounds which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing compounds.

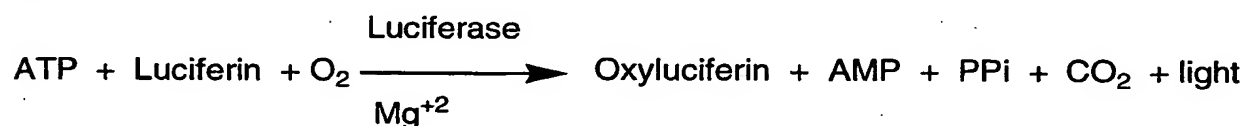
#### IN VITRO CELL PROLIFERATION ASSAYS

Generally, the cytotoxic or cytostatic activity of an antibody-drug conjugate (ADC) is measured by: exposing mammalian cells having tumor-associated antigens or receptor proteins to the antibody of the ADC in a cell culture medium; culturing the cells for a period from about 6 hours to about 5 days; and measuring cell viability. Cell-based *in vitro* assays were used to measure viability, i.e. proliferation ( $\text{IC}_{50}$ ), cytotoxicity ( $\text{EC}_{50}$ ), and induction of apoptosis (caspase activation) of the ADC.

The *in vitro* potency of antibody-drug conjugates was measured by a cell proliferation assay (Figures 1-4). The CellTiter-Glo® Luminescent Cell Viability Assay is a commercially available (Promega Corp., Madison, WI), homogeneous assay method based on the recombinant expression of *Coleoptera* luciferase (US 5583024; US 5674713; US 5700670). This cell proliferation assay determines the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells (Crouch et al (1993) J. Immunol. Meth. 160:81-88; US 6602677). The CellTiter-Glo® Assay was conducted in 96 well format,

making it amenable to automated high-throughput screening (HTS) (Cree et al (1995) AntiCancer Drugs 6:398-404). The homogeneous assay procedure involves adding the single reagent (CellTiter-Glo® Reagent) directly to cells cultured in serum-supplemented medium. Cell washing, removal of medium and multiple pipetting steps are not required. The system detects as few as 15 cells/well in a 384-well format in 10 minutes after adding reagent and mixing.

The homogeneous "add-mix-measure" format results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of cells present in culture. The CellTiter-Glo® Assay generates a "glow-type" luminescent signal, produced by the luciferase reaction, which has a half-life generally greater than five hours, depending on cell type and medium used. Viable cells are reflected in relative luminescence units (RLU). The substrate, Beetle Luciferin, is oxidatively decarboxylated by recombinant firefly luciferase with concomitant conversion of ATP to AMP and generation of photons. The extended half-life eliminates the need to use reagent injectors and provides flexibility for continuous or batch mode processing of multiple plates. This cell proliferation assay can be used with various multiwell formats, e.g. 96 or 384 well format. Data can be recorded by luminometer or CCD camera imaging device. The luminescence output is presented as relative light units (RLU), measured over time.



The anti-proliferative effects of three antibody-drug conjugates were measured by the cell proliferation, *in vitro* cell killing assay above against four different breast tumor cell lines (Figures 1-4). Figure 1 shows the potency measurements at increasing concentrations of trastuzumab-SPP-DM1, trastuzumab-SPDP-DM1 and trastuzumab-SMCC-DM1 after a 3 day treatment on SK-BR-3 (HER2 3+) breast tumor cells. Figure 2 shows the potency measurements of at increasing concentrations of trastuzumab-SPP-DM1, trastuzumab-SPDP-DM1 and trastuzumab-SMCC-DM1 after a 3 day treatment on BT-474 (HER2 3+) breast tumor cells. Figure 3 shows the potency measurements of at increasing concentrations of trastuzumab-SPP-DM1, trastuzumab-SPDP-DM1 and trastuzumab-SMCC-DM1 after a 3 day treatment on MCF7 (HER2 low) breast tumor cells. Figure 4 shows the potency measurements at increasing concentrations of trastuzumab-SPP-DM1, trastuzumab-SPDP-DM1 and trastuzumab-SMCC-DM1 after a 3 day treatment on MDA-MB-468 (HER2 negative) breast tumor cells.

IC<sub>50</sub> values were established for SK-BR-3 and BT-474 which are known to overexpress HER2 receptor protein. A lot of conjugate trastuzumab-SPP-DM1 with 2.8 DM1 per trastuzumab (drug/Ab) gave a mean IC<sub>50</sub> of 14.4 µg/ml with a range 9.1 to 22.3 µg/ml for 6 experiments against SK-BR-3 cells and a mean IC<sub>50</sub> of 51.7 µg/ml with a range 28.7 to 63.1 µg/ml for 4 experiments against BT-474 cells. A lot of conjugate trastuzumab-SMCC-DM1 with 2.7 DM1 per trastuzumab (drug/Ab) gave a mean IC<sub>50</sub> of 15.2 µg/ml with a range 12.6 to 18.8 µg/ml for 4 experiments against SK-BR-3 cells and a mean IC<sub>50</sub> of 94.9 µg/ml with a range 75.2 to 114.6 µg/ml for 2 experiments against BT-474 cells. The conjugates were inactive against cells MCF7 and MDA-MB-468 which do not overexpress HER2.

AntiCD19-SMCC-DM1 showed potent cell killing *in vitro* with Raji cells ( $IC_{50} = <0.25 \mu\text{g/ml}$ ) where the naked antibody and control ADC, trastuzumab-SMCC-DM1 showed no effect. AntiCD79a-SMCC-DM1 and antiCD79b-SMCC-DM1 showed potent cell killing *in vitro* with Ramos cells ( $IC_{50} = <0.25 \mu\text{g/ml}$ ) where the naked antibody and control ADC, trastuzumab-SMCC-DM1 showed no effect.

Figure 17 shows an *in vitro*, cell proliferation assay (Example 5) with HT1080EphB2 (C8) cells treated with antiEphB2R 2H9 antibody-drug conjugates: 2H9-SPP-DM1 ( $IC_{50}$  80 ng/ml), and 2H9-SMCC-DM1 ( $IC_{50}$  50 ng/ml).

#### IN VIVO SERUM CLEARANCE AND STABILITY IN MICE

Serum clearance and stability of ADC were investigated in nude, naive (without tumors received by exogenous grafts) mice. Figure 5 shows the serum clearance in beige nude mice without tumors of trastuzumab-SMCC-DM1 vs. trastuzumab-SPP-DM1, measuring conjugate and total antibody serum concentration at six time points over 7 days. A difference in the amount of total antibody and ADC indicates cleavage of the linker and separation of the antibody from its DM1 moiety. As illustrated by the higher conjugated antibody serum concentration at seven days, SMCC-linked ADC remained intact *in vivo* longer than did SPP-linked conjugates.

Figure 6 shows the stability over time in nude mice without tumors of the conjugates: trastuzumab-SPDP-DM1, trastuzumab-SPP-DM1, trastuzumab-SPP-DM3, trastuzumab-SPP-DM4, and trastuzumab-SMCC-DM1, measuring serum concentration at six time points over 7 days. ADC with the SMCC linker were more stable *in vivo* than were conjugates linked by SPP or SPDP, although trastuzumab-SMCC-DM1 had approximately the same stability as the most hindered disulfide conjugate, trastuzumab-SPP-DM4.

The experiments shown in Figures 5 and 6 were performed in nude mice without tumors. However, SMCC-linked conjugates showed the same increased stability compared to SPP-linked conjugates in nude mice with tumors. As shown in Figure 7, approximately 72% of the initial trastuzumab-SMCC-DM1 remained as a conjugate 7 days after treatment, while only approximately 10% of the initial trastuzumab-SPP-DM1 remained as a conjugate 7 days after treatment, illustrating the improved stability of non-enzymatically cleavable trastuzumab-SMCC-DM1 conjugates in nude mice with tumors.

#### IN VIVO SERUM CLEARANCE AND STABILITY IN RATS

Figures 8 and 9 show the comparative stability and clearance profiles of a disulfide linker ADC (trastuzumab-SPP-DM1 and a non-disulfide linker ADC (trastuzumab-SMCC-DM1) in rats. The parameters of the study include:

Parameter	trastuzumab-SPP-DM1	trastuzumab-SMCC-DM1
Vd (ml/kg)	41	41
Clearance (ml/day/kg)	52	15
T ½ alpha	0.09	0.15
T ½ beta	0.7	0.85
T ½ gamma (days)	2.6	5.5

The non-disulfide linker ADC, trastuzumab-SMCC-DM1 (Figure 9), showed better stability in rat serum than the disulfide linker ADC, trastuzumab-SPP-DM1 (Figure 8).

#### IN VIVO EFFICACY

5 The efficacy of the antibody-drug conjugates of the invention may be measured *in vivo* by implanting allografts or xenografts of cancer cells in rodents and treating the tumors with ADC. Variable results are to be expected depending on the cell line, the specificity of antibody binding of the ADC to receptors present on the cancer cells, dosing regimen, and other factors. The *in vivo* efficacy of anti-HER2 ADC was measured by a high expressing HER2 transgenic explant mouse model. An allograft was propagated from the Fo5 mmtv  
10 transgenic mouse which does not respond to, or responds poorly to, HERCEPTIN therapy. Subjects were treated once with ADC and monitored over 3-6 weeks to measure the time to tumor doubling, log cell kill, and tumor shrinkage. Follow up dose-response and multi-dose experiments were conducted.

15 Tumors arise readily in transgenic mice that express a mutationally activated form of *neu*, the rat homolog of HER2, but the HER2 that is overexpressed in breast cancers is not mutated and tumor formation is much less robust in transgenic mice that overexpress nonmutated HER2 (Webster et al (1994) Semin. Cancer Biol. 5:69-76).

20 To improve tumor formation with nonmutated HER2, transgenic mice were produced using a HER2 cDNA plasmid in which an upstream ATG was deleted in order to prevent initiation of translation at such upstream ATG codons, which would otherwise reduce the frequency of translation initiation from the downstream authentic initiation codon of HER2 (for example, see Child et al (1999) J. Biol. Chem. 274: 24335-24341). Additionally, a chimeric intron was added to the 5' end, which should also enhance the level of expression as reported earlier (Neuberger and Williams (1988) Nucleic Acids Res. 16: 6713; Buchman and Berg (1988) Mol. Cell. Biol. 8: 4395; Brinster et al (1988) Proc. Natl. Acad. Sci. USA 85:836). The chimeric intron was derived from a Promega vector, pCI-neo mammalian expression vector (bp 890-1022). The cDNA 3'-end is  
25 flanked by human growth hormone exons 4 and 5, and polyadenylation sequences. Moreover, FVB mice were used because this strain is more susceptible to tumor development. The promoter from MMTV-LTR was used to ensure tissue-specific HER2 expression in the mammary gland. Animals were fed the AIN 76A diet in order to increase susceptibility to tumor formation (Rao et al (1997) Breast Cancer Res. and Treatment 45:149-158).

30 Figures 10-13 show that the ADC have strong anti-tumor activity in the allograft of a HER2 positive tumor (Fo5) that originally arose in an MMTV-HER2 transgenic mouse. The antibody alone (e.g. trastuzumab) does not have significant anti-tumor activity in this model (Erickson et al US 6632979). As illustrated in

Figures 10 and 11, the growth of the tumors was retarded by treatment with ADC as compared to control (Vehicle) level of growth. Tumor growth was slowed most by treatment with trastuzumab-SMCC-DM1 and trastuzumab-SLAB-DM1 conjugates. As shown in Figures 10, 12 and 13, the trastuzumab-SMCC-DM1 conjugate slowed tumor growth more than conjugates with SPP linkers, i.e. more potency, whether measured as doubling time of tumors in nude mice or as Log Cell Kill corresponding to the doubling time measurements shown (Figure 13).

The *in vivo* efficacy of anti-CD22 ADC was measured with a mouse tumor xenograft model. Groups of eight SCID mice with 20 million Bjab-luc (luciferase expressing Bjab cells) xenograft tumor cells per mouse were dosed once at day 1 (except where noted) with an anti-CD22 antibody-drug conjugate, or a naked antibody (Example 8).

ADC or Ab	$\mu\text{g DM1/m}^2$	Ab mg / kg mouse	average drug loading (p)	MTD (days)
Tr-SMCC-DM1	200	4.2	3.2	3
7A2-SMCC-DM1	200	3.8	3.6	6
5E8-SMCC-DM1	200	3.8	3.6	10
RFB4-SMCC-DM1	200	3.2	4.25	18
RFB4-SMCC-DM1 (dosed 3X at 1, 7, 14 days)	405	10	2.75	55
7A2	-	4	-	3
5E8	-	4	-	3
RFB4	-	4	-	3

The time for tumor size to double was measured (MTD, mean tumor doubling time). The three naked anti-CD22 antibodies showed essentially no efficacy relative to a non-specific binding ADC (trastuzumab-SMCC-DM1). The corresponding conjugates all showed the effect of significantly retarding tumor growth. The effect of multiple dosing was established with RFB4-SMCC-DM1 where the MTD for singly dosed mice was 18 days whereas the MTD for mice dosed three times, at days 1, 7, and 14, was 55 days. Complete remission of tumor occurred in all 8 mice in the triple-dosed group. Other antiCD22-SMCC-DM1 conjugates, with antibodies 12F7, 9A8, 8C9, 8G10, 3F11, 10D2, 6C9, 14D1, and 11H10, showed shrinkage of initial tumor volume or retardation of tumor growth relative to control (trastuzumab-SMCC-DM1), after 7 days following a single dose (400  $\mu\text{g DM1/m}^2$ ) in SCID mice with 20 million Bjab-luc xenograft tumor cells per mouse. AntiCD22 conjugates, RFB4-SMCC-DM1, 5E8-SMCC-DM1, and 7A2-SMCC-DM1 were also effective in retardation of tumor growth relative to control (trastuzumab-SMCC-DM1), after 11 days following a single dose (200  $\mu\text{g DM1/m}^2$ ) in SCID mice with 5 million Ramos RA1 xenograft tumor cells per mouse.

The conjugate RFB4-SMCC-DM1 was studied at three different drug loadings on groups of ten SCID mice with Bjab-luc xenografts (Example 8). The low (1.95) and medium (3.7) drug loaded conjugates each

showed the effect of significantly retarding tumor growth, with an MTD of about 15 days. The high loaded (6.75) conjugate did not show an effect significantly different than control conjugate GP120-SMCC-DM1, or naked antibody RFB4.

anti-CD22 ADC or Ab	$\mu\text{g DM1/m}^2$	Ab mg / kg mouse	average drug loading (p)	MTD (days)
RFB4	-	10	-	3
RFB4-SMCC-DM1 (low loaded)	144	5	1.95	15
RFB4-SMCC-DM1 (medium loaded)	273	5	3.7	15
RFB4-SMCC-DM1 (high loaded)	497	5	6.75	3
GP120-SMCC-DM1 (high loaded)	449	5	6.1	3

5 AntiCD19-SMCC-DM1 and antiCD22-SMCC-DM1 conjugates did not show *in vivo* activity in a Raji cell mouse tumor xenograft model. Other antiCD19 and antiCD22 conjugates may have *in vivo* activity against other cancer cell tumor models.

10 The *in vivo* efficacy of anti-CD79a (alpha) and anti-CD79b (beta) ADC was measured with a mouse tumor xenograft model. Groups of eight SCID mice with 20 million Bjab-luc xenograft tumor cells per mouse were dosed at day 1 with samples in the table below and following Example 8.

ADC, Ab, or control	$\mu\text{g DM1/m}^2$	Ab mg / kg mouse	average drug loading (p)	MTD (days)
PBS (buffer control)	-	-	-	3.5
antiGP120	-	3.2	-	3.5
SN8 antiCD79b	-	3.1	-	4
17A7 antiCD79b	-	3.1	-	4
8H9 antiCD79a	-	4.0	-	3
antiGP120-SMCC-DM1	200	3.2	4.2	3.5
SN8 antiCD79b-SMCC-DM1	200	3.1	4.4	>7
17A7 antiCD79b-SMCC-DM1	200	3.1	4.4	>7
8H9 antiCD79a-SMCC-DM1	200	4.0	3.4	>7

Conjugates SN8 antiCD79b-SMCC-DM1, 17A7 antiCD79b-SMCC-DM1, and 8H9 antiCD79a-SMCC-DM1, all showed shrinkage of initial tumor volume (mean 160 mm<sup>3</sup>) after 7 days. In the groups of 8 mice, conjugate SN8 antiCD79b-SMCC-DM1 gave partial remission (PR) in 4 animals and complete remission (CR) in 2 animals. Conjugate 17A7 antiCD79b-SMCC-DM1 gave CR in 1 animal. Conjugate 8H9 antiCD79a-SMCC-DM1 gave PR in 2 animals and CR in 1 animal. Other antiCD79b-SMCC-DM1 conjugates, with antibodies 2F2, 5C3, 7H7, 8D11, 15E4, and 16C11, showed retardation of tumor growth or shrinkage of initial tumor volume, relative to control (trastuzumab-SMCC-DM1), after 8 days following a single dose (192 µg DM1/m<sup>2</sup>) in CB17 ICR SCID mice with 20 million Bjab-luc xenograft tumor cells per mouse.

The dose response effect on mice administered with antiCD79b-SMCC-DM1 was measured. Groups of eight SCID mice with 20 million Bjab-luc xenograft tumor cells per mouse were dosed at day 1 with samples in the table below (Example 8). The antiCD79b-SMCC-DM1 was dosed at levels of 0.5, 2.0, and 3.64 mg Ab/kg mouse.

ADC, Ab, or control	µg DM1/m <sup>2</sup>	Ab mg / kg mouse	MTD (days)
Vehicle control (PBS)	-		4
antiCD79b-SMCC-DM1	32	0.5	10
antiCD79b-SMCC-DM1	130	2.0	35
antiCD79b-SMCC-DM1	236	3.64	>70

The *in vivo* efficacy of antiTENB2 ADC was measured with a mouse tumor xenograft model. TENB2 is a tumor antigen that is shown to be almost exclusively expressed in the human prostate and overexpressed in human prostate tumors (Glynne-Jones et al (2001) Int J Cancer. Oct 15;94(2):178-84). PC3-TVA-919cv1:5 is a human prostate cancer cell line expressing high level of TENB2.

Athymic nude mice were subcutaneously injected with 5 million PC3-TVA-919 high expressor or medium expressor cells in a volume of 0.2 ml per mouse. Cells were suspended in HBSS. When mean tumor size reached 100-200 mm<sup>3</sup>, the mice were randomly grouped into 8 groups of 8-10 mice each and give a single IV treatment of the samples below (Example 8).

ADC or control	$\mu\text{g DM1/m}^2$	Ab mg / kg mouse	medium expressor MTD (days)	high expressor MTD (days)
PBS (vehicle control)	-	-	30	22
murine anti-ragweed-SPP-DM1 (negative control)	373	6.68	18	22
murine anti-ragweed-SMCC-DM1 (negative control)	373	9.34	14	18
murine antiTENB2:3146-SPP-DM1	373	7.34	58	35
murine antiTENB2:3146-SMCC-DM1	373	10.6	44	19
chimeric antiTENB2-SPP-DM1	373	8.7	57	43
chimeric antiTENB2-SMCC-DM1	373	7.63	43	22

Murine anti-TENB2-DM1 conjugates showed anti-tumor efficacy against PC3-TENB2 tumors, relative to negative control and vehicle control. Murine 10H1 antiNaPi3b-SMCC-DM1 conjugate showed no anti-tumor efficacy against PC3-NaPi3b tumors, relative to negative control and vehicle control. Other antibody variants of antiNaPi3b conjugates may have in vivo activity against PC3-NaPi3b tumors, or other cancer cell lines.

#### RODENT TOXICITY

Antibody-drug conjugates and an ADC-minus control, "Vehicle", were evaluated in an acute toxicity rat model. Toxicity of ADC was investigated by treatment of female Sprague-Dawley rats with the ADC and subsequent inspection and analysis of the effects on various organs. Based on gross observations (body weights), clinical pathology parameters (serum chemistry and hematology) and histopathology, the toxicity of ADC may be observed, characterized, and measured. It was found that at equivalent dose levels, trastuzumab-SMCC-DM1 was associated with less acute toxicity than trastuzumab-SPP-DM1.

A 5-day acute toxicity study in adolescent female rats (100-125 gms) was conducted by a single injection of trastuzumab-SMCC-DM1 (two doses: 1860 and 3260  $\mu\text{g DM1/m}^2$ ), a comparison disulfide ADC, trastuzumab-SPP-DM1 (two doses: 1860 and 3260  $\mu\text{g DM1/m}^2$ ), free DM1 maytansine (thiol) and a control Vehicle (day 0). Body weight was measured daily. Clinical chemistry, serum enzymes and hematology analysis was conducted on days 3 and 5; concluding with complete necropsy with histopathological assessment. Toxicity signals included the clinical observation of weight loss.

It is considered that weight loss, or weight change relative to animals dosed only with Vehicle in animals after dosing with ADC, is a gross and general indicator of systemic or localized toxicity. Figure 14 shows the changes in body weight (grams) over 5 days. Rats receiving the disulfide ADC, trastuzumab-SPP-DM1 showed a marked, dose-dependent toxicity, indicated by lethality at the higher dose and a decline in body

weight at the lower dose. In contrast, rats receiving trastuzumab-SMCC-DM1 gained weight, with the lower dosed rats showing no decline in the rate of weight gain, relative to the placebo Vehicle-dosed rats. Rats dosed at the higher level of trastuzumab-SMCC-DM1 also gained weight, comparable to the free DM1 cytotoxin.

Hepatotoxicity was measured by elevated liver enzymes, increased numbers of mitotic and apoptotic figures and hepatocyte necrosis. Hematolymphoid toxicity was observed by depletion of leukocytes, primarily granulocytes (neutrophils), and/or platelets, and lymphoid organ involvement, i.e. atrophy or apoptotic activity. Toxicity was also noted by gastrointestinal tract lesions such as increased numbers of mitotic and apoptotic figures and degenerative enterocolitis.

Enzymes indicative of liver injury that were studied include:

AST (aspartate aminotransferase)

–Localization: cytoplasmic; liver, heart, skeletal muscle, kidney

–Liver:Plasma ratio of 7000:1

–T1/2: 17 hrs

ALT (alanine aminotransferase)

–Localization: cytoplasmic; liver, kidney, heart, skeletal muscle

–Liver:Plasma ratio of 3000:1

–T1/2: 42 hrs; diurnal variation

GGT (g-glutamyl transferase)

–Localization: plasma membrane of cells with high secretory or absorptive capacity; liver, kidney, intestine

–Poor predictor of liver injury; commonly elevated in bile duct disorders

None of the three enzymes measured above are liver-specific. It was found that the ADC of the present invention caused a transient, mild elevation of liver enzymes ALT and AST, with transient reticulocytopenia (Figure 15). No significant effect on peripheral blood granulocytes or platelets was observed (Figure 16).

Figures 15 and 16 show that rats exposed to 22.3 mg/kg trastuzumab-SPP-DM1, (Group 2) demonstrated the most severe clinical toxicity in this five day acute toxicity study. These animals showed the most profound loss of body weight, elevations of liver function tests, leuko- and thrombocytopenia and morphologic evidence of toxicity against hematolymphoid tissues. The extent of toxicity was similar compared to that encountered in previous studies using a dose of 25mg/kg. In contrast, animals in Groups 3 and 4 given trastuzumab-SMCC-DM1 at 10 and 25 mg/kg, respectively, were indistinguishable from vehicle-treated animals based on clinical pathology and body weight data. Morphologically, these animals showed a mildly increased number of mitotic figures in the liver, however, peripheral lymphoid and hematopoietic tissues were within normal limits.

Group 5 animals, 50 mg/kg trastuzumab-SMCC-DM1, showed evidence of toxicity. However, with the exception of one liver function test (ALT), the severity of toxicity was less than in animals receiving 50% of the same drug dose as trastuzumab-SPP-DM1 (Group 2). At about the same dose, trastuzumab-SMCC-DM1 (Group 4, 22.3 mg/kg) showed about 25% of the AST level as trastuzumab-SPP-DM1 (Group 2, 25 mg/kg). By day 5 of this study, Group 5 animals showed increases in body weights (following a transitory loss during days 3 and 4), a decreasing serum bilirubin and a rising platelet count (Figure 15).

Animals exposed to free maytansinoid DM1 (Group 6) show the same pattern of toxicity as animals treated with trastuzumab conjugates. This dose of free DM1 corresponds to the amount of drug given as a 10 mg/kg trastuzumab-SPP-DM1 dose in previous studies. The severity of toxicity in animals of group 6 was less than that seen in animals of Group 2, but more than previously seen in animals treated with 10 mg/kg trastuzumab-SPP-DM1. Recovery appeared to be rather fast: The sections of spleen showed increased numbers of immature hematopoietic elements in animals treated with free maytansine; also, LFTs and clinical hematology parameters showed a distinct trend towards normalization in animals of Group 6 by day 5.

#### CYNOMOLGUS MONKEY TOXICITY/SAFETY

Toxicity and safety of ADC administered to Cynomolgus monkeys may be assessed. A toxicity/safety study of the antibody-drug conjugate, trastuzumab-SMCC-DM1, was conducted in Cynomolgus monkeys. Three groups of monkeys were studied to assess the toxicity of trastuzumab-SMCC-DM1 administered via intravenous injection at escalating doses, relative to control (Vehicle). Group 1 (4 subjects) received only Vehicle (PBS, pH 6.5, i.e. formulation minus ADC) at day 1 and day 22, followed by necropsy at day 36. Group 2 (4 subjects) received trastuzumab-SMCC-DM1 4900  $\mu\text{g}/\text{m}^2$  at day 1 and at day 22. Group 3 (4 subjects) received trastuzumab-SMCC-DM1 7200  $\mu\text{g}/\text{m}^2$  at day 22.

Hepatotoxicity was inferred by measurement of the elevated liver enzymes from the Rodent Toxicity study. Cynomolgus monkeys were dosed with Vehicle (Group 1) and trastuzumab-SMCC-DM1 (Group 2: 4900  $\mu\text{g}/\text{m}^2$ ; Group 3: 7200  $\mu\text{g}/\text{m}^2$ ). Liver enzyme AST, platelet counts, white blood cells, absolute neutrophil, red blood cells, reticulocytes, and a comparison of 2 IV dose regimens were measured for trastuzumab-SMCC-DM1 in Cynomolgus Monkeys (Example 10).

#### ADMINISTRATION OF ANTIBODY-DRUG CONJUGATE PHARMACEUTICAL FORMULATIONS

Therapeutic antibody-drug conjugates (ADC) may be administered by any route appropriate to the condition to be treated. The ADC will typically be administered parenterally, i.e. infusion, subcutaneous, intramuscular, intravenous, intradermal, intrathecal, bolus, intratumor injection or epidural (Shire et al (2004) J. Pharm. Sciences 93(6):1390-1402). Pharmaceutical formulations of therapeutic antibody-drug conjugates (ADC) are typically prepared for parenteral administration with a pharmaceutically acceptable parenteral vehicle and in a unit dosage injectable form. An antibody-drug conjugate (ADC) having the desired degree of purity is optionally mixed with pharmaceutically acceptable diluents, carriers, excipients or stabilizers, in the form of a lyophilized formulation or an aqueous solution (Remington's Pharmaceutical Sciences (1980) 16th edition, Osol, A. Ed.).

Acceptable parenteral vehicles, diluents, carriers, excipients, and stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose,

mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). For example, lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein by reference. An exemplary formulation of an ADC such as trastuzumab-SMCC-DM1 contains about 100 mg/ml of trehalose (2-(hydroxymethyl)-6-[3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]oxy-tetrahydropyran-3,4,5-triol; C<sub>12</sub>H<sub>22</sub>O<sub>11</sub>; CAS Number 99-20-7) and about 0.1% TWEEN™ 20 (polysorbate 20; dodecanoic acid 2-[2-[3,4-bis(2-hydroxyethoxy)tetrahydrofuran-2-yl]-2-(2-hydroxyethoxy)ethoxy]ethyl ester; C<sub>26</sub>H<sub>50</sub>O<sub>10</sub>; CAS Number 9005-64-5) at approximately pH 6.

Pharmaceutical formulations of a therapeutic antibody-drug conjugate (ADC) may contain certain amounts of unreacted drug moiety (D), antibody-linker intermediate (Ab-L), and/or drug-linker intermediate (D-L), as a consequence of incomplete purification and separation of excess reagents, impurities, and by-products, in the process of making the ADC; or time/temperature hydrolysis or degradation upon storage of the bulk ADC or formulated ADC composition. For example, a formulation of the ADC, trastuzumab-SMCC-DM1 may contain a detectable amount of free drug DM1. Alternatively, or in addition to, it may contain a detectable amount of drug-linker intermediate DM1-SMCC. Alternatively, or in addition to, it may contain a detectable amount of the antibody, trastuzumab. An exemplary formulation of trastuzumab-SMCC-DM1 may contain up to 10% molar equivalent of DM1-SMCC. Unexpectedly, it was determined by the *in vitro* cellular proliferation assay (Example 5), that DM1-SMCC (IC<sub>50</sub> 0.05 μM) is about 20 times less potent in cell killing than free drug DM1 (IC<sub>50</sub> 0.0045 μM) against SK-BR-3 and BT-474 breast cancer cells.

The active pharmaceutical ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semi permeable matrices of solid hydrophobic polymers containing the ADC, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (US 3773919), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

The formulations to be used for *in vivo* administration must be sterile, which is readily accomplished by filtration through sterile filtration membranes.

The formulations include those suitable for the foregoing administration routes. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Techniques and formulations generally are found in *Remington's Pharmaceutical Sciences* (Mack Publishing Co., Easton, PA). Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared

by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Aqueous suspensions contain the active materials (ADC) in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients include a suspending agent, such as sodium carboxymethylcellulose, croscarmellose, povidone, methylcellulose, hydroxypropyl methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia, and dispersing or wetting agents such as a naturally occurring phosphatide (e.g., lecithin), a condensation product of an alkylene oxide with a fatty acid (e.g., polyoxyethylene stearate), a condensation product of ethylene oxide with a long chain aliphatic alcohol (e.g., heptadecaethyleneoxycetanol), a condensation product of ethylene oxide with a partial ester derived from a fatty acid and a hexitol anhydride (e.g., polyoxyethylene sorbitan monooleate). The aqueous suspension may also contain one or more preservatives such as ethyl or n-propyl p-hydroxy-benzoate, one or more coloring agents, one or more flavoring agents and one or more sweetening agents, such as sucrose or saccharin.

The pharmaceutical compositions of ADC may be in the form of a sterile injectable preparation, such as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents which have been mentioned above. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, such as a solution in 1,3-butane-diol or prepared as a lyophilized powder. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile fixed oils may conventionally be employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid may likewise be used in the preparation of injectables.

The amount of active ingredient that may be combined with the carrier material to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. For example, an aqueous solution intended for intravenous infusion may contain from about 3 to 500  $\mu$ g of the active ingredient per milliliter of solution in order that infusion of a suitable volume at a rate of about 30 mL/hr can occur. Subcutaneous (bolus) administration may be effected with about 1.5 ml or less of total volume and a concentration of about 100 mg ADC per ml. For ADC that require frequent and chronic administration, the subcutaneous route may be employed, such as by pre-filled syringe or autoinjector device technology.

As a general proposition, the initial pharmaceutically effective amount of ADC administered per dose will be in the range of about 0.01-100 mg/kg, namely about 0.1 to 20 mg/kg of patient body weight per day, with the typical initial range of compound used being 0.3 to 15 mg/kg/day. For example, human patients may be initially dosed at about 1.5 mg ADC per kg patient body weight. The dose may be escalated to the maximally tolerated dose (MTD). The dosing schedule may be about every 3 weeks, but according to diagnosed condition or response, the schedule may be more or less frequent. The dose may be further adjusted during the course of treatment to be at or below MTD which can be safely administered for multiple cycles, such as about 4 or more.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents.

Although oral administration of protein therapeutics are generally disfavored due to poor bioavailability due to limited absorption, hydrolysis or denaturation in the gut, formulations of ADC suitable for oral administration may be prepared as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the ADC.

5 The formulations may be packaged in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water, for injection immediately prior to use. Extemporaneous injection solutions and suspensions are prepared from sterile powders, granules and tablets of the kind previously described. Exemplary unit dosage formulations contain a daily dose or unit daily sub-dose, or an appropriate fraction thereof, of the active ingredient.

10 The invention further provides veterinary compositions comprising at least one active ingredient as above defined together with a veterinary carrier therefore. Veterinary carriers are materials useful for the purpose of administering the composition and may be solid, liquid or gaseous materials which are otherwise inert or acceptable in the veterinary art and are compatible with the active ingredient. These veterinary compositions may be administered parenterally, orally or by any other desired route.

#### ANTIBODY-DRUG CONJUGATE TREATMENTS

It is contemplated that the antibody-drug conjugates (ADC) of the present invention may be used to treat various diseases or disorders, such as cancer and autoimmune conditions. Exemplary conditions or disorders include benign or malignant tumors; leukemia and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic, glandular, macrophagal, epithelial, stromal, blastocoelec, inflammatory, angiogenic and immunologic disorders. Cancer susceptible to ADC treatment include those which are characterized by the overexpression of certain tumor associated antigens or cell surface receptors, e.g. HER2

20 The ADC compounds which are identified in the animal models and cell-based assays can be further tested in tumor-bearing higher primates and human clinical trials. Human clinical trials can be designed similar to the clinical trials testing the efficacy of the anti-HER2 monoclonal antibody HERCEPTIN in patients with HER2 overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy as reported by Baselga et al. (1996) J. Clin. Oncol. 14:737-744. The clinical trial may be designed to evaluate the efficacy of an ADC in combination with known therapeutic regimens, such as radiation and/or chemotherapy involving known chemotherapeutic and/or cytotoxic agents (Pegram et al (1999) Oncogene 18:2241-2251).

30 Generally, the disease or disorder to be treated is cancer. Examples of cancer to be treated herein include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g. epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, gastrointestinal stromal tumor (GIST), pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, as well as head and neck cancer.

The cancer to be treated herein may be one characterized by excessive activation of an ErbB receptor, e.g. HER2. Such excessive activation may be attributable to overexpression or increased production of the ErbB receptor or an ErbB ligand. In one embodiment, a diagnostic or prognostic assay will be performed to determine whether the patient's cancer is characterized by excessive activation of an ErbB receptor. For example, ErbB gene amplification and/or overexpression of an ErbB receptor in the cancer may be determined. Various assays for determining such amplification/overexpression are available in the art and include the IHC, FISH and shed antigen assays described above. Alternatively, or additionally, levels of an ErbB ligand, such as TGF- $\alpha$ , in or associated with the tumor may be determined according to known procedures. Such assays may detect protein and/or nucleic acid encoding it in the sample to be tested. In one embodiment, ErbB ligand levels in the tumor may be determined using immunohistochemistry (IHC); see, for example, Scher et al. (1995) Clin. Cancer Research 1:545-550. Alternatively, or additionally, one may evaluate levels of ErbB ligand-encoding nucleic acid in the sample to be tested; e.g. via FISH, southern blotting, or PCR techniques. In one embodiment, ErbB2 overexpression may be analyzed by IHC, e.g. using the HERCEPTEST (Dako). Paraffin embedded tissue sections from a tumor biopsy may be subjected to the IHC assay and accorded a ErbB2 protein staining intensity criteria as follows: Score 0, no staining is observed or membrane staining is observed in less than 10% of tumor cells; Score 1+, a faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells, the cells are only stained in part of their membrane; Score 2+, a weak to moderate complete membrane staining is observed in more than 10% of the tumor cells; Score 3+, a moderate to strong complete membrane staining is observed in more than 10% of the tumor cells. Those tumors with 0 or 1+ scores for ErbB2 overexpression assessment may be characterized as not overexpressing ErbB2, whereas those tumors with 2+ or 3+ scores may be characterized as overexpressing ErbB2.

Alternatively, or additionally, FISH assays such as the INFORM™ (Ventana Co., Ariz.) or PATHVISION™ (Vysis, Ill.) may be carried out on formalin-fixed, paraffin-embedded tumor tissue to determine the extent (if any) of ErbB2 overexpression in the tumor.

Moreover, ErbB receptor or ErbB ligand overexpression or amplification may be evaluated using an *in vivo* diagnostic assay, e.g. by administering a molecule (such as an antibody) which binds the molecule to be detected and is tagged with a detectable label (e.g. a radioactive isotope) and externally scanning the patient for localization of the label.

For the prevention or treatment of disease, the appropriate dosage of an ADC will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the molecule is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The molecule is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1  $\mu\text{g/kg}$  to 15  $\text{mg/kg}$  (e.g. 0.1-20  $\text{mg/kg}$ ) of molecule is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1  $\mu\text{g/kg}$  to 100  $\text{mg/kg}$  or more, depending on the factors mentioned above. An exemplary dosage of ADC to be administered to a patient is in the range of about 0.1 to about 10  $\text{mg/kg}$  of patient weight.

For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. An exemplary dosing regimen comprises

administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the anti-ErbB2 antibody. Other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

#### COMBINATION THERAPY

5 An antibody-drug conjugate (ADC) may be combined in a pharmaceutical combination formulation, or dosing regimen as combination therapy, with a second compound having anti-cancer properties. The second compound of the pharmaceutical combination formulation or dosing regimen preferably has complementary activities to the ADC of the combination such that they do not adversely affect each other.

10 The second compound may be a chemotherapeutic agent, cytotoxic agent, cytokine, growth inhibitory agent, anti-hormonal agent, aromatase inhibitor, protein kinase inhibitor, lipid kinase inhibitor, anti-androgen, antisense oligonucleotide, ribozyme, gene therapy vaccine, anti-angiogenic agent and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended. A pharmaceutical composition containing an ADC may also have a therapeutically effective amount of a chemotherapeutic agent such as a tubulin-forming inhibitor, a topoisomerase inhibitor, or a DNA binder.

15 Alternatively, or additionally, the second compound may be an antibody which binds ErbB2 and blocks ligand activation of an ErbB receptor. The second antibody may be monoclonal antibody 2C4 or humanized 2C4 "Omnitarg" (WO 01/00245). The second antibody may be conjugated with a cytotoxic or chemotherapeutic agent, e.g., a maytansinoid, an auristatin, a calicheamicin, or a 1,8 bis-naphthalimide moiety. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2, ErbB3, ErbB4, or  
20 vascular endothelial factor (VEGF) in the one formulation or dosing regimen.

Other therapeutic regimens may be combined with the administration of an anticancer agent identified in accordance with this invention. The combination therapy may be administered as a simultaneous or sequential regimen. When administered sequentially, the combination may be administered in two or more administrations. The combined administration includes coadministration, using separate formulations or a  
25 single pharmaceutical formulation, and consecutive administration in either order, wherein there is a time period while both (or all) active agents simultaneously exert their biological activities.

In one embodiment, treatment with an ADC of the present invention involves the combined administration of an anticancer agent identified herein, and one or more chemotherapeutic agents or growth inhibitory agents, including coadministration of cocktails of different chemotherapeutic agents, optionally along  
30 with treatment with an anti-ErbB2 antibody, such as trastuzumab. Chemotherapeutic agents include Erlotinib HCl (CP-358774, TARCEVA™; Genentech/OSI), taxanes (such as paclitaxel and doxorubicin) and/or anthracycline antibiotics. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers's instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M.C. Perry,  
35 Williams & Wilkins, Baltimore, Md. (1992).

The anticancer agent may be combined with an anti-hormonal compound; e.g., an anti-estrogen compound such as tamoxifen; an anti-progesterone such as onapristone (EP 616812); or an anti-androgen such as flutamide, in dosages known for such molecules. Where the cancer to be treated is hormone independent cancer, the patient may previously have been subjected to anti-hormonal therapy and, after the cancer becomes  
40 hormone independent, the anti-ErbB2 antibody (and optionally other agents as described herein) may be

administered to the patient. It may be beneficial to also coadminister a cardioprotectant (to prevent or reduce myocardial dysfunction associated with the therapy) or one or more cytokines to the patient. In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

5           Suitable dosages for any of the above coadministered agents are those presently used and may be lowered due to the combined action (synergy) of the newly identified agent and other chemotherapeutic agents or treatments.

10           The combination therapy may provide "synergy" and prove "synergistic", i.e. the effect achieved when the active ingredients used together is greater than the sum of the effects that results from using the compounds separately. A synergistic effect may be attained when the active ingredients are: (1) co-formulated and administered or delivered simultaneously in a combined, unit dosage formulation; (2) delivered by alternation or in parallel as separate formulations; or (3) by some other regimen. When delivered in alternation therapy, a synergistic effect may be attained when the compounds are administered or delivered sequentially, e.g. by different injections in separate syringes. In general, during alternation therapy, an effective dosage of each  
15           active ingredient is administered sequentially, i.e. serially, whereas in combination therapy, effective dosages of two or more active ingredients are administered together.

#### METABOLITES OF THE ANTIBODY-DRUG CONJUGATES

20           Also falling within the scope of this invention are the *in vivo* metabolic products of the ADC compounds described herein, to the extent such products are novel and unobvious over the prior art. Such products may result for example from the oxidation, reduction, hydrolysis, amidation, esterification, enzymatic cleavage, and the like, of the administered compound. Accordingly, the invention includes novel and unobvious compounds produced by a process comprising contacting a compound of this invention with a mammal for a period of time sufficient to yield a metabolic product thereof.

25           Metabolite products may be identified by preparing a radiolabelled (e.g.  $^{14}\text{C}$  or  $^3\text{H}$ ) ADC, administering it parenterally in a detectable dose (e.g. greater than about 0.5 mg/kg) to an animal such as rat, mouse, guinea pig, monkey, or to man, allowing sufficient time for metabolism to occur (typically about 30 seconds to 30 hours) and isolating its conversion products from the urine, blood or other biological samples. These products are easily isolated since they are labeled (others are isolated by the use of antibodies capable of binding epitopes surviving in the metabolite). The metabolite structures are determined in conventional fashion,  
30           e.g. by MS, LC/MS or NMR analysis. In general, analysis of metabolites is done in the same way as conventional drug metabolism studies well-known to those skilled in the art. The conversion products, so long as they are not otherwise found *in vivo*, are useful in diagnostic assays for therapeutic dosing of the ADC compounds.

35           Metabolites include the products of *in vivo* cleavage of the ADC where cleavage of any bond occurs that links the drug moiety to the antibody. Metabolic cleavage may thus result in the naked antibody, or an antibody fragment. The antibody metabolite may be linked to a part, or all, of the linker. Metabolic cleavage may also result in the production a drug moiety or part thereof. The drug moiety metabolite may be linked to a part, or all, of the linker.

#### ARTICLES OF MANUFACTURE

In another embodiment, an article of manufacture, or "kit", containing ADC and materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, or blister pack. The containers may be formed from a variety of materials such as glass or plastic. The container holds an antibody-drug conjugate (ADC) composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an ADC. The label or package insert indicates that the composition is used for treating the condition of choice, such as cancer. In one embodiment, the label or package inserts indicates that the composition comprising the antibody which binds ErbB2 can be used to treat cancer which expresses an ErbB receptor selected from the group consisting of epidermal growth factor receptor (EGFR), ErbB2, ErbB3 and ErbB4. In addition, the label or package insert may indicate that the patient to be treated is one having cancer characterized by excessive activation of an ErbB receptor selected from EGFR, ErbB2, ErbB3 or ErbB4. For example, the cancer may be one which overexpresses one of these receptors and/or which overexpresses an ErbB ligand (such as TGF- $\alpha$ ). The label or package insert may also indicate that the composition can be used to treat cancer, wherein the cancer is not characterized by overexpression of the ErbB2 receptor. In other embodiments, the package insert may indicate that the ADC composition can be used also to treat hormone independent cancer, prostate cancer, colon cancer or colorectal cancer.

The article of manufacture may comprise (a) a first container with a compound contained therein, wherein the compound comprises an ADC of the present invention in which the antibody of the ADC is a first antibody which binds ErbB2 and inhibits growth of cancer cells which overexpress ErbB2; and (b) a second container with a compound contained therein, wherein the compound comprises a second antibody which binds ErbB2 and blocks ligand activation of an ErbB receptor, or a conjugate of this second antibody with a maytansinoid. The article of manufacture in this embodiment may further comprise a package insert indicating that the first and second compounds can be used to treat cancer. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

#### EXAMPLES

##### Example 1 - Production, Characterization and Humanization of Anti-ErbB2 Monoclonal Antibody 4D5

The murine monoclonal antibody 4D5 which specifically binds the extracellular domain of ErbB2 was produced as described in Fendly et al (1990) Cancer Research 50:1550-1558. Briefly, NIH 3T3/HER2-3400 cells (expressing approximately  $1 \times 10^5$  ErbB2 molecules/cell) produced as described in Hudziak et al (1987) Proc. Natl. Acad. Sci. (USA) 84:7158-7163 were harvested with phosphate buffered saline (PBS) containing 25mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of  $10^7$  cells in 0.5ml PBS on weeks 0, 2, 5 and 7. The mice with antisera that immunoprecipitated  $^{32}$ P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13.

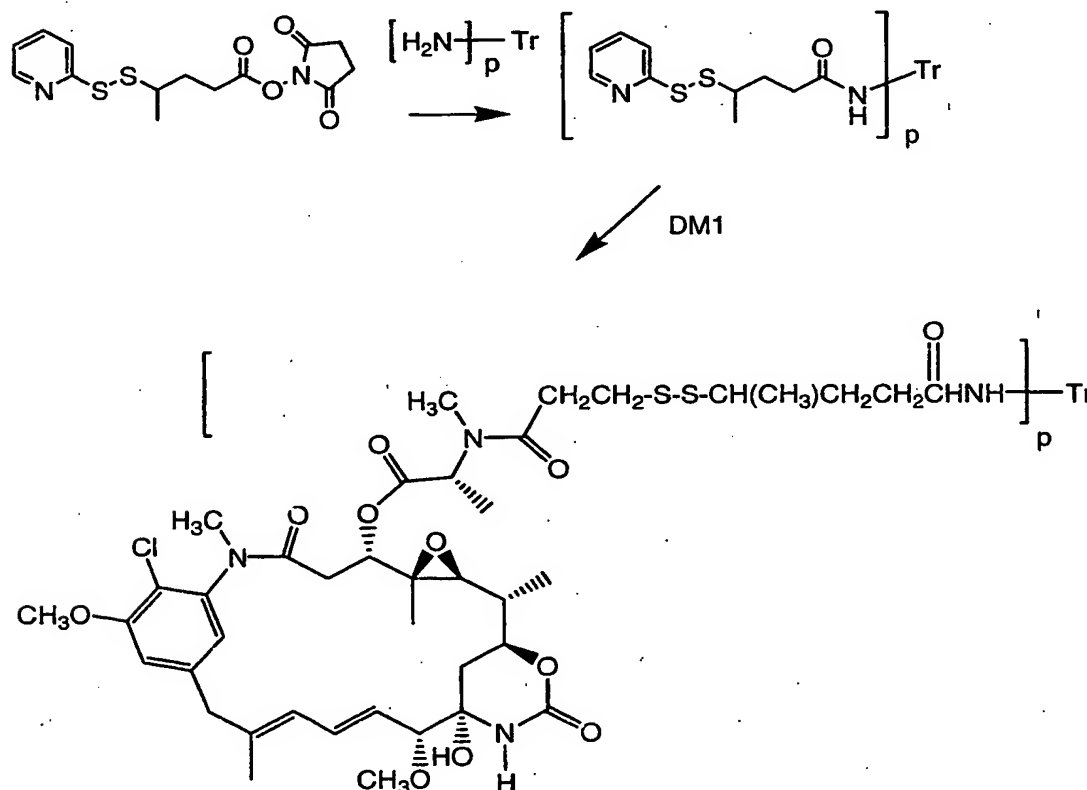
This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation.

The murine monoclonal antibody 4D5 was humanized, using a "gene conversion mutagenesis" strategy, as described in US 5821337, the entire disclosure of which is hereby expressly incorporated by reference. The humanized monoclonal antibody 4D5 used in the following experiments is designated huMAb4D5-8. This antibody is of IgG1 isotype.

#### Example 2 - Purification of Trastuzumab

One vial containing 440 mg HERCEPTIN® (huMAb4D5-8, rhuMAb HER2, US 5821337) antibody) was dissolved in 50 mL MES buffer (25 mM MES, 50 mM NaCl, pH 5.6) and loaded on a cation exchange column (Sephacrose S, 15 cm x 1.7 cm) that had been equilibrated in the same buffer. The column was then washed with the same buffer (5 column volumes). Trastuzumab was eluted by raising the NaCl concentration of the buffer to 200 mM. Fractions containing the antibody were pooled, diluted to 10 mg/mL, and dialyzed into a buffer containing 50 mM potassium phosphate, 50 mM NaCl, 2 mM EDTA, pH 6.5.

#### Example 3 - Preparation of Antibody-Drug Conjugates: Trastuzumab-SPP-DM1:



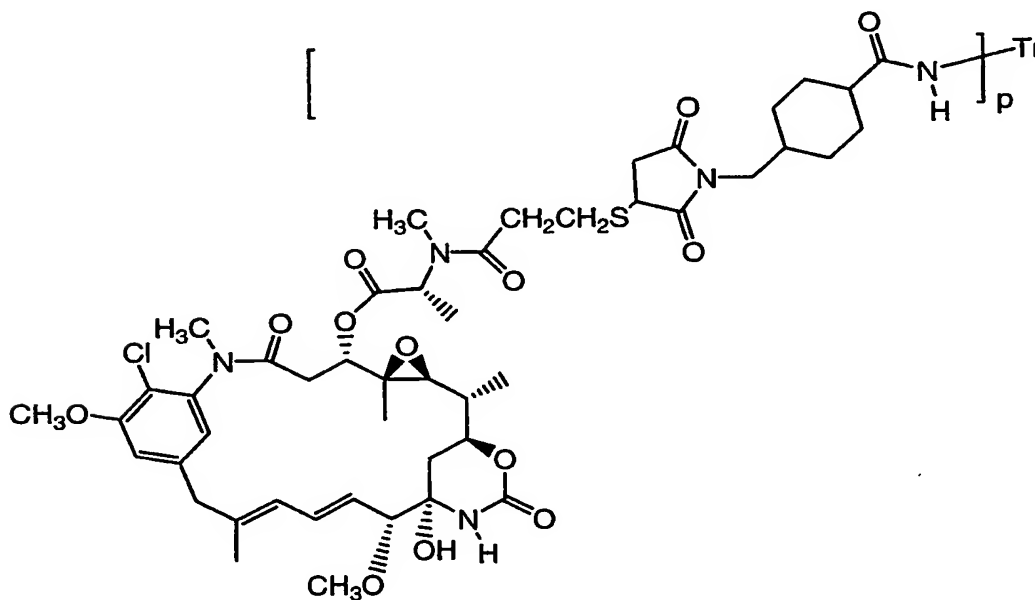
Purified trastuzumab was derivatized with N-succinimidyl-4-(2-pyridylthio)pentanoate to introduce dithiopyridyl groups. Trastuzumab (376.0 mg, 8 mg/mL) in 44.7 mL of 50 mM potassium phosphate buffer (pH 6.5) containing NaCl (50 mM) and EDTA (1 mM) was treated with SPP (5.3 molar equivalents in 2.3 mL

ethanol). After incubation for 90 minutes under argon at ambient temperature, the reaction mixture was gel filtered through a Sephadex G25 column equilibrated with 35 mM sodium citrate, 154 mM NaCl, 2 mM EDTA. Antibody containing fractions were pooled and assayed. The degree of modification of the antibody was determined as described above. Recovery of the modified antibody (trastuzumab-SPP-Py) was 337 mg (89.7%) with 4.5 releasable 2-thiopyridine groups linked per antibody (p').

Trastuzumab-SPP-Pv (1.70 mg, 9.5  $\mu$ mol of releasable 2-thiopyridine groups) was diluted with the above 35 mM sodium phosphate buffer, pH 6.5, to a final concentration of 2.5 mg/mL. DM1 (N<sup>2</sup>-deacetyl-N<sup>2</sup>-(3-maytansine), the structure of which is shown in Figure 1, (1.7 equivalents, 16.1  $\mu$ mol) was added to the final reaction mixture) was then added to the antibody solution. The reaction mixture was stirred at room temperature under argon for 20 hours.

The reaction mixture was loaded on a Sephacryl S300 gel filtration column (5.0 cm x 90.0 cm, 1.77 L) equilibrated with 0.1 M sodium citrate, 154 mM NaCl, pH 6.5. The flow rate was 5.0 mL/min and 65 fractions were collected. The major peak centered around fraction No. 47 (Figure 3). The major peak fractions (44-51) were pooled and assayed. The number of DM1 drug molecules linked per antibody molecule ( $p'$ ) was determined by measuring the absorbance at 252 nm and 280 nm, and found to be 3.7 drug molecules per antibody molecule.

#### File 4 - Preparation of Antibody-Drug Conjugates: Trastuzumab-SMCC-DM1



Purified trastuzumab was derivatized with (Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate, SMCC, Pierce Biotechnology, Inc) to introduce the SMCC linker. Trastuzumab was purified from HERCEPTIN® as in Example 2 and buffer-exchange treated at 20 mg/mL in 50 mM potassium phosphate/ 50 mM sodium chloride/ 2 mM EDTA, pH 6.5 with 7.5 to 10 molar equivalents of SMCC (20 mM in DMSO or DMA (dimethylacetamide), 6.7 mg/mL). After stirring for 2 to 4 hours under argon at ambient temperature, the reaction mixture was filtered through a Sephadex G25 column equilibrated with 50mM potassium phosphate/ 50 mM sodium chloride/ 2 mM EDTA, pH 6.5. Alternatively, the reaction mixture was gel filtered with 30 mM

citrate and 150 mM sodium chloride at pH 6. Antibody containing fractions were pooled and assayed. Recovery of trastuzumab-SMCC was 88%.

5 The drug-linker intermediate, trastuzumab-SMCC from above, was diluted with 50 mM potassium phosphate/50 mM sodium chloride/2 mM EDTA, pH 6.5, to a final concentration of 10 mg/ml, and reacted with a 10 mM solution of DM1 (1.7 equivalents assuming 5 SMCC/trastuzumab, 7.37 mg/ml) in dimethylacetamide. The reaction was stirred at ambient temperature under argon for 4 to about 16 hours. The conjugation reaction mixture was filtered through a Sephadex G25 gel filtration column (1.5 x 4.9 cm) with 1 x PBS at pH 6.5. Alternatively, the reaction mixture was gel filtered with 10 mM succinate and 150 mM sodium chloride at pH 5. The DM1/trastuzumab ratio (p) was 3.1, as measured by the absorbance at 252 nm and at 280 nm. The drug to antibody ratio (p) may also be measured by mass spectrometry. Conjugation may also be monitored by SDS polyacrylamide gel electrophoresis. Aggregation may be assessed by laser light scattering analysis.

#### Antibody-SMCC-DM1 Conjugates:

Following this protocol, other antibody-drug conjugates with the SMCC linker and DM1 drug moiety were prepared, including:

Antibody-drug conjugate (ADC)	antibody (Ab)	antigen	average drug loading (DM1/Ab)
10H-SMCC-DM1	10H1 (murine) anti-NaPi3b	NaPi3b	5.0
2H9-SMCC-DM1	2H9 Mab	anti-EphB2R	4.0
ragweed-SMCC-DM1	ragweed		2.7
antiTENB2-SMCC-DM1	anti-TENB2-3416#2 Mouse MAb	TENB2	2.5
anti-TENB2 20D1 Fc Chimera-SMCC-DM1	Chimeric Human Mouse anti-TENB2	TENB2	3.32
antiCD19-SMCC-DM1	antiCD19 (mIgG1 isotype)	CD19	5.05
2H7-SMCC-DM1	antiCD20 (mIgG2a isotype)	CD20	2.44, 3.84 (two lots)
CD20 LC-SMCC-DM1	antiCD20 (Rituxan®)	CD20	
HB5-SMCC-DM1	antiCD21 (mIgG2a isotype)	CD21	4.05
RFB4-SMCC-DM1	antiCD22	CD22	1.95-6.75 (multiple lots)
Cy34.1.2-SMCC-DM1	antiCD22	CD22	2.70
12F7.1.5-SMCC-DM1	antiCD22	CD22	5.90
9A8.1.1-SMCC-DM1	antiCD22	CD22	5.00

8C9.1.2-SMCC-DM1	antiCD22	CD22	2.90
14B3.3.1-SMCC-DM1	antiCD22	CD22	5.20
8G10.4.2-SMCC-DM1	antiCD22	CD22	3.60
2B4.1.4-SMCC-DM1	antiCD22	CD22	5.20
7A2.4.1-SMCC-DM1	antiCD22	CD22	3.60
4H3.2.2-SMCC-DM1	antiCD22	CD22	3.60
5E8.1.8-SMCC-DM1	antiCD22	CD22	3.60
3F11.2.1-SMCC-DM1	antiCD22	CD22	4.50
6C9.1.3-SMCC-DM1	antiCD22	CD22	3.70
10D2.4.3-SMCC-DM1	antiCD22	CD22	3.65
10D6.8.1-SMCC-DM1	antiCD72	CD72	2.90
ZL7-SMCC-DM1	antiCD79a (alpha)	CD79a	3.60
5C3-SMCC-DM1	antiCD79a (alpha)	CD79a	1.85
6G1-SMCC-DM1	antiCD79a (alpha)	CD79a	3.50
7H7-SMCC-DM1	antiCD79a (alpha)	CD79a	3.25
8D11-SMCC-DM1	antiCD79a (alpha)	CD79a	3.60
8H9-SMCC-DM1	antiCD79a (alpha)	CD79a	3.40
11E5-SMCC-DM1	antiCD79a (alpha)	CD79a	3.60
15E4-SMCC-DM1	antiCD79a (alpha)	CD79a	3.25
16C11-SMCC-DM1	antiCD79a (alpha)	CD79a	2.50
2F2-SMCC-DM1	antiCD79b (beta)	CD79b	3.20
17A7-SMCC-DM1	antiCD79b (beta)	CD79b	4.40
SN8-SMCC-DM1	antiCD79b (beta)	CD79b	4.40, 0.80 (two lots)
1F9-SMCC-DM1	antiFcRH1	FcRH1	4.20
7A2-SMCC-DM1	antiFcRH2	FcRH2	4.20, 3.95 (two lots)
7G7-SMCC-DM1	antiFcRH2	FcRH2	3.80
c11D6-SMCC-DM1	antiFcRH2	FcRH2	2.20
51505.111-SMCC-DM1	antiCXCR5	CXCR5	6.10

12C7 Fc Chimera-SMCC-DM1	antiBrevican	brevican	4.60
H2:PSCA hlog-SMCC-DM1	antiPSCA hlog	PSCA	3
H6:xPSCAhlog-SMCC-DM1	antiPSCA hlog	PSCA	2.6
xFcRH5:3909 7D11.1.1-SMCC-DM1	antiIRTA2	IRTA2	2.6
xFcRH5.7D11-SMCC-DM1	antiIRTA2	IRTA2	4.4
11D10 LC-SMCC-DM1	Mouse anti-CA 0772P MAb	CA 0772P MUC16	
xNCA LC-SMCC-DM1	Mouse anti-CEACAM6 MAb	CEACAM6 NCA	

#### Antibody-BMPEO-DM1 Conjugates:

For cysteine conjugation, the antibody may be reduced with a reducing agent such as dithiothreitol (DTT) or TCEP, in the presence of EDTA. After incubating for about one hour at 37 °C, the pH is adjusted to about 7 with 100 mM potassium phosphate. The reduced antibody is modified by the bis-maleimido reagent BM(PEO)<sub>4</sub> (Pierce Chemical), leaving an unreacted maleimido group on the surface of the antibody. This may be accomplished by dissolving BM(PEO)<sub>4</sub> in a 50% ethanol/water mixture to a concentration of 10 mM and adding a tenfold molar excess to a solution containing antibody in phosphate buffered saline at a concentration of approximately 1.6 mg/ml (10 µM) and allowing it to react for 1 hour to form Ab-BMPEO. Excess BM(PEO)<sub>4</sub> is removed by gel filtration (HiTrap column, Pharmacia) in 30 mM citrate, pH 6 with 150 mM NaCl buffer. An approximate 10 fold molar excess DM1 is dissolved in dimethyl acetamide (DMA) and added to the Ab-BMPEO intermediate. Dimethyl formamide (DMF) may also be employed to dissolve the drug moiety reagent. The reaction mixture is allowed to react overnight before gel filtration or dialysis into PBS to remove unreacted DM1. Gel filtration on S200 columns in PBS was used to remove high molecular weight aggregates and furnish purified Ab-BMPEO-DM1.

Following this protocol, other antibody-drug conjugates with the BMPEO linker and DM1 drug moiety were prepared, including:

Antibody-drug conjugate (ADC)	antibody (Ab)	antigen	average drug loading (DM1/Ab)
trastuzumab-BM(PEO)-DM1	trastuzumab (Herceptin®)	HER2	2.94
CD120-BMPEO4-DM1	Mouse anti-GP120 MAb	GP120	2
RFB4-BMPEO4-DM1	antiCD22	CD22	3.7 and 4.25 (two lots)

Example 5 - In vitro cell proliferation assay

Efficacy of ADC were measured by a cell proliferation assay employing the following protocol (Promega Corp. Technical Bulletin TB288; Mendoza et al (2002) Cancer Res. 62:5485-5488):

1. An aliquot of 100  $\mu$ l of cell culture containing about  $10^4$  cells (SKBR-3, BT474, MCF7 or MDA-MB-468) in medium was deposited in each well of a 96-well, opaque-walled plate.
2. Control wells were prepared containing medium and without cells.
3. ADC was added to the experimental wells and incubated for 3-5 days.
4. The plates were equilibrated to room temperature for approximately 30 minutes.
5. A volume of CellTiter-Glo Reagent equal to the volume of cell culture medium present in each well was added.
6. The contents were mixed for 2 minutes on an orbital shaker to induce cell lysis.
7. The plate was incubated at room temperature for 10 minutes to stabilize the luminescence signal.
8. Luminescence was recorded and reported in graphs as RLU = relative luminescence units.

Example 6 - Serum clearance and stability in mice

A beige mutation, nude, naive (without tumors) mice in six Groups of four animals were studied. At Day 0, each mouse received a single 2 mg/kg dose of ADC in 200  $\mu$ l aqueous carrier, except the Vehicle Group which received only carrier. Blood was collected by cardiac puncture under anesthesia at each time point (5 minutes, 1 hour, 6 hours, 24 hours, 72 hours, and 168 hours) post-dose. The serum was isolated and antibody and ADC were measured.

Group 1: Vehicle (PBS, pH 6.5, no ADC)

Group 2: Trastuzumab-SMCC-DM1

Group 3: Trastuzumab-SPP-DM1

Group 4: Trastuzumab-SPDP-DM1

Group 5: Trastuzumab-SPP-DM3

Group 5: Trastuzumab-SPP-DM4

Example 7 - Serum stability in rats

Six dose groups with 6 Sprague-Dawley rats (100-125 gms each) per dose group were studied. At Day 0, Animals were administered a single IV dose of Vehicle, 10 mg/kg trastuzumab-SPP-DM1, or 10 mg/kg trastuzumab-SMCC-DM1, at a dose volume of 10 ml/kg via the lateral tail vein. Approximately 300  $\mu$ l whole blood was collected at each timepoint: 0 (predose), 10, and 30 minutes; 1, 2, 4, 8, 24 and 36 hours; and 2, 3, 4, 7, 14, 21, 28 days post dose.

Example 8 - Tumor volume in vivo efficacy

High expressing HER2 transgenic explant mice:

Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Taconic (Germantown, N.Y.). Many strains are suitable, but FVB female mice are preferred because of their higher susceptibility to tumor formation. FVB males were used for mating and vasectomized CD.1 studs were used to stimulate pseudopregnancy. Vasectomized mice can be obtained from any commercial supplier.

Founders were bred with either FVB mice or with 129/BL6 x FVB p53 heterozygous mice. The mice with heterozygosity at p53 allele were used to potentially increase tumor formation. However, this has proven unnecessary. Therefore, some F1 tumors are of mixed strain. Founder tumors are FVB only. Six founders were obtained with some developing tumors without having litters.

- 5           Animals having tumors (allograft propagated from Fo5 mmtv transgenic mice) were treated with a single injection of a trastuzumab-DM1 maytansinoid conjugate (10 mg/kg dose) and tumor volume was assessed for over 20 days after injection.

Bjab-luc xenograft SCID mice:

- 10           Groups of eight to ten SCID mice with 20 million Bjab-luc xenograft tumor cells per mouse were dosed at day 1 with an antibody-drug conjugate, or a naked antibody. A group of eight mice were tested each with anti-CD22 ADC, naked anti-CD22 antibodies, and control. Control ADC (trastuzumab-SMCC-DM1; loading: DM1/trastuzumab = 3.2) is not a specific binder and was administered at 200 µg DM1/m<sup>2</sup>, 4.2 trastuzumab/kg mouse, and resulted in a mean tumor doubling time of about 3 days. Anti-CD22 conjugates 7A2-SMCC-DM1 (loading: DM1/Ab = 3.6), 5E8-SMCC-DM1 (loading: DM1/Ab = 3.6), and RFB4-SMCC-DM1 (loading: DM1/Ab = 4.3). Naked antibodies, 7A2, 5E8, and RFB4 were dosed at 4 mg/kg mouse.
- 15

#### Example 9 - Rat toxicity

- 20           The acute toxicity profile of trastuzumab-SPP-DM1 (disulfide linker) compared with that of free DM1 and trastuzumab-SMCC-DM1 (non-disulfide linker) was evaluated. Animals were injected on day 1, complete chemistry and hematology profiles were obtained at baseline, day 3 and day 5 and a complete necropsy was performed on day 5. Routine histology was performed on three random animals for each group for the following tissues: sternum, liver, kidney, thymus, spleen, large and small intestine. The experimental groups were as follows:

- 25           Group 1: Vehicle (10 mM sodium succinate, 100 mg/mL sucrose, 0.1% Tween 20, pH 5.0)  
             Group 2: Trastuzumab-SPP-DM1, 22.3mg/kg  
             Group 3: Trastuzumab-SMCC-DM1, 10mg/kg  
             Group 4: Trastuzumab-SMCC-DM1, 25mg/kg  
             Group 5: Trastuzumab-SMCC-DM1, 50mg/kg  
             Group 6: Free DM1, 160µg/kg

#### Example 10 - Cynomolgus Monkey Toxicity/Safety

- 30           Three groups of four (2 male, 2 female) naive *Macaca fascicularis* (cynomolgus monkey) were studied.

            Group 1: (4 animals) received only Vehicle (PBS, pH 6.5, i.e. formulation minus ADC) at day 1 and day 22, followed by necropsy at day 36.

            Group 2: (4 animals) received trastuzumab-SMCC-DM1 4900 µg/m<sup>2</sup> at day 1 and at day 22.

- 35           Group 3: (4 animals) received trastuzumab-SMCC-DM1 7200 µg/m<sup>2</sup> at day 22.

Dosing is expressed in surface area of an animal so as to be relevant to other species, i.e. dosage at µg/m<sup>2</sup> is independent of species and thus comparable between species. Formulations of trastuzumab-SMCC-

DM1 for Group 2 and Group 3 studies contained PBS, 5.4mM sodium phosphate, 4.2 mM potassium phosphate, 140 mM sodium chloride, pH 6.5.

5 Blood was collected for hematology analysis prior to first dosing (acclimation period) and at days 3, 7, 11, and 14 after the first dose (Groups 1 and 2) and at days 3, 7, 11, 14, and 21 after the second dose (Groups 1, 2 and 3). Erythrocyte (RBC) and platelet (PLT) counts were measured by the light scattering method. Leukocyte (WBC) count was measured by the peroxidase/basophil method. Reticulocyte count was measured by the light scattering method with cationic dye. Cell counts were measured on an Advia 120 apparatus. ALT (alanine aminotransferase) and AST (aspartate aminotransferase) were measured in U/L by UV/NADH; IFCC methodology on an Olympus AU400 apparatus, and using Total Ab ELISA - ECD/GxhuFc-HRP. Conj. Ab  
10 ELISA - xDM1/ECD-Bio/SA-HRP tests.

All patents, patent applications, and references cited throughout the specification are expressly incorporated by reference.

We claim:

1. An antibody-drug conjugate compound comprising an antibody covalently attached by a linker to one or more maytansinoid drug moieties, the compound having Formula I:



or a pharmaceutically acceptable salt or solvate thereof, wherein:

Ab is an antibody which binds to an ErbB receptor, or which binds to one or more tumor-associated antigens or cell-surface receptors selected from (1)-(36):

(1) BMPR1B (bone morphogenetic protein receptor-type IB, Genbank accession no. NM\_001203);

(2) E16 (LAT1, SLC7A5, Genbank accession no. NM\_003486);

(3) STEAP1 (six transmembrane epithelial antigen of prostate, Genbank accession no. NM\_012449);

(4) 0772P (CA125, MUC16, Genbank accession no. AF361486);

(5) MPF (MPF, MSLN, SMR, megakaryocyte potentiating factor, mesothelin, Genbank accession no. NM\_005823);

(6) Napi3b (NAPI-3B, NPT1b, SLC34A2, solute carrier family 34 (sodium phosphate), member 2, type II sodium-dependent phosphate transporter 3b, Genbank accession no. NM\_006424);

(7) Sema 5b (FLJ10372, KIAA1445, Mm.42015, SEMA5B, SEMAG, Semaphorin 5b Hlog, sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5B, Genbank accession no. AB040878);

(8) PSCA hlg (2700050C12Rik, C530008O16Rik, RIKEN cDNA 2700050C12, RIKEN cDNA 2700050C12 gene, Genbank accession no. AY358628);

(9) ETBR (Endothelin type B receptor, Genbank accession no. AY275463);

(10) MSG783 (RNF124, hypothetical protein FLJ20315, Genbank accession no. NM\_017763);

(11) STEAP2 (HGNC\_8639, IPCA-1, PCANAP1, STAMP1, STEAP2, STMP, prostate cancer associated gene 1, prostate cancer associated protein 1, six transmembrane epithelial antigen of prostate 2, six transmembrane prostate protein, Genbank accession no. AF455138);

(12) TrpM4 (BR22450, FLJ20041, TRPM4, TRPM4B, transient receptor potential cation channel, subfamily M, member 4, Genbank accession no. NM\_017636);

(13) CRIPTO (CR, CR1, CRGF, CRIPTO, TDGF1, teratocarcinoma-derived growth factor, Genbank accession no. NP\_003203 or NM\_003212);

(14) CD21 (CR2 (Complement receptor 2) or C3DR (C3d/Epstein Barr virus receptor) or Hs.73792 Genbank accession no. M26004);

(15) CD79b (CD79B, CD79β, Ig (immunoglobulin-associated beta), B29, Genbank accession no. NM\_000626);

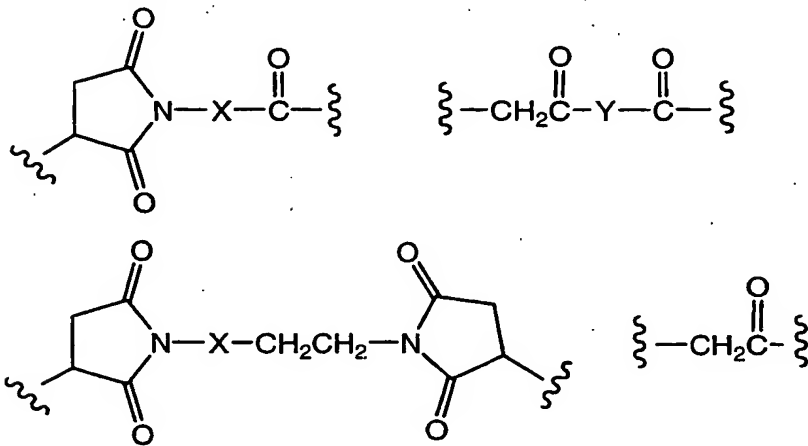
(16) FcRH2 (IFGP4, IRTA4, SPAP1A (SH2 domain containing phosphatase anchor protein 1a), SPAP1B, SPAP1C, Genbank accession no. NM\_030764);

(17) HER2 (Genbank accession no. M11730);

- (18) NCA (Genbank accession no. M18728);
- (19) MDP (Genbank accession no. BC017023);
- (20) IL20R $\alpha$  (Genbank accession no. AF184971);
- (21) Brevican (Genbank accession no. AF229053);
- 5 (22) EphB2R (Genbank accession no. NM\_004442);
- (23) ASLG659 (Genbank accession no. AX092328);
- (24) PSCA (Genbank accession no. AJ297436);
- (25) GEDA (Genbank accession no. AY260763);
- (26) BAFF-R (B cell -activating factor receptor, BLyS receptor 3, BR3, NP\_443177.1);
- 10 (27) CD22 (B-cell receptor CD22-B isoform, NP-001762.1);
- (28) CD79a (CD79A, CD79 $\alpha$ , immunoglobulin-associated alpha, a B cell-specific protein that covalently interacts with Ig beta (CD79B) and forms a complex on the surface with Ig M molecules, transduces a signal involved in B-cell differentiation, Genbank accession No. NP\_001774.1);
- (29) CXCR5 (Burkitt's lymphoma receptor 1, a G protein-coupled receptor that is activated by the
- 15 CXCL13 chemokine, functions in lymphocyte migration and humoral defense, plays a role in HIV-2 infection and perhaps development of AIDS, lymphoma, myeloma, and leukemia, Genbank accession No. NP\_001707.1);
- (30) HLA-DOB (Beta subunit of MHC class II molecule (Ia antigen) that binds peptides and presents them to CD4+ T lymphocytes, Genbank accession No. NP\_002111.1);
- 20 (31) P2X5 (Purinergic receptor P2X ligand-gated ion channel 5, an ion channel gated by extracellular ATP, may be involved in synaptic transmission and neurogenesis, deficiency may contribute to the pathophysiology of idiopathic detrusor instability, Genbank accession No. NP\_002552.2);
- (32) CD72 (B-cell differentiation antigen CD72, Lyb-2, Genbank accession No. NP\_001773.1);
- (33) LY64 (Lymphocyte antigen 64 (RP105), type I membrane protein of the leucine rich repeat (LRR)
- 25 family, regulates B-cell activation and apoptosis, loss of function is associated with increased disease activity in patients with systemic lupus erythematosus, Genbank accession No. NP\_005573.1);
- (34) FcRH1 (Fc receptor-like protein 1, a putative receptor for the immunoglobulin Fc domain that contains C2 type Ig-like and ITAM domains, may have a role in B-lymphocyte differentiation, Genbank accession No. NP\_443170.1);
- 30 (35) IRTA2 (Immunoglobulin superfamily receptor translocation associated 2, a putative immunoreceptor with possible roles in B cell development and lymphomagenesis; deregulation of the gene by translocation occurs in some B cell malignancies, Genbank accession No. NP\_112571.1); and
- (36) TENB2 (putative transmembrane proteoglycan, related to the EGF/heregulin family of growth
- 35 factors and follistatin, Genbank accession No. AF179274;
- provided that the antibody is not TA.1;
- L is a non-disulfide linker;
- D is a maytansinoid drug moiety; and
- p is 1 to 8.

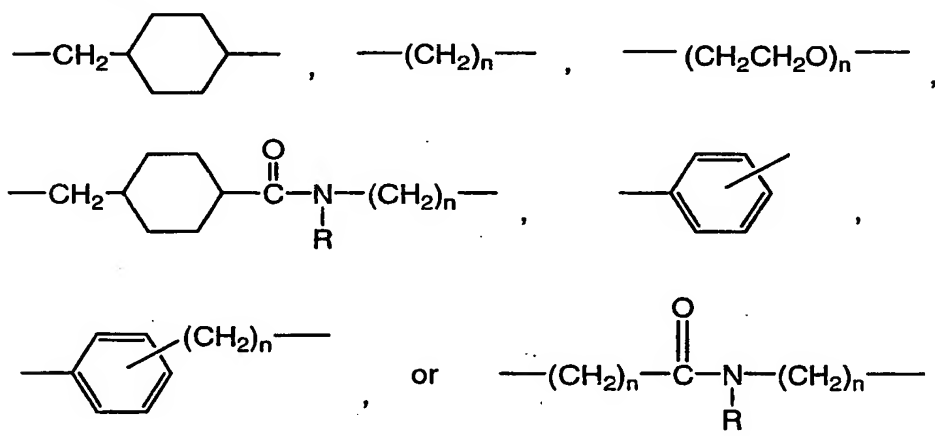
2. The antibody-drug conjugate compound of claim 1 wherein Ab is an antibody which binds to one or more tumor-associated antigens or cell-surface receptors selected from (1)-(16) and (18)-(36).

3. The antibody-drug conjugate compound of claim 1 or claim 2 wherein L is a linker selected from the structures:

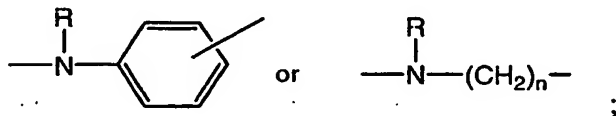


where the wavy lines indicate the covalent attachments to Ab and D;

X is:

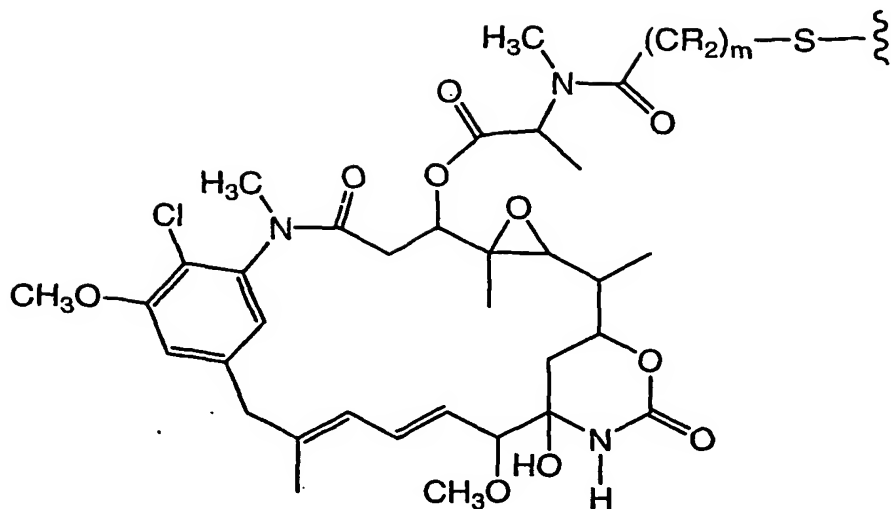


Y is:



R is independently H or C<sub>1</sub>-C<sub>6</sub> alkyl; and n is 1 to 12.

4. The antibody-drug conjugate compound of any of the preceding claims wherein D is selected from the structure:



where the wavy line indicates the covalent attachment to L,

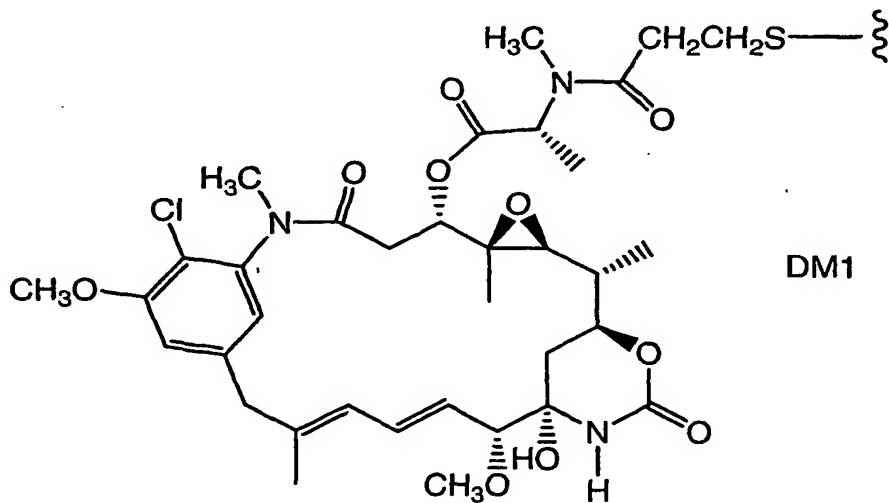
R is independently H or C<sub>1</sub>-C<sub>6</sub> alkyl, and

m is 1, 2, or 3.

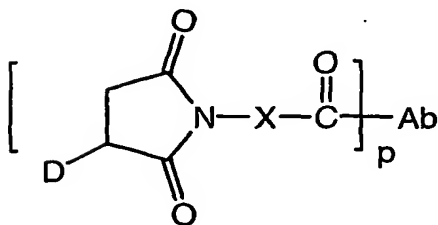
5

5. The antibody-drug conjugate compound of claim 4 wherein m is 2, and R is H.

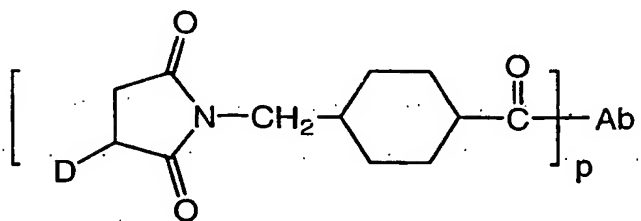
6. The antibody-drug conjugate compound of any of the preceding claims wherein the maytansinoid drug moiety is DM1, having the structure:



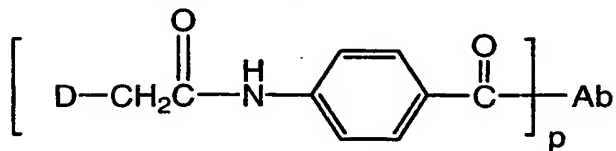
7. The antibody-drug conjugate compound of claim 3 having the structure:



8. The antibody-drug conjugate compound of claim 7 having the structure:

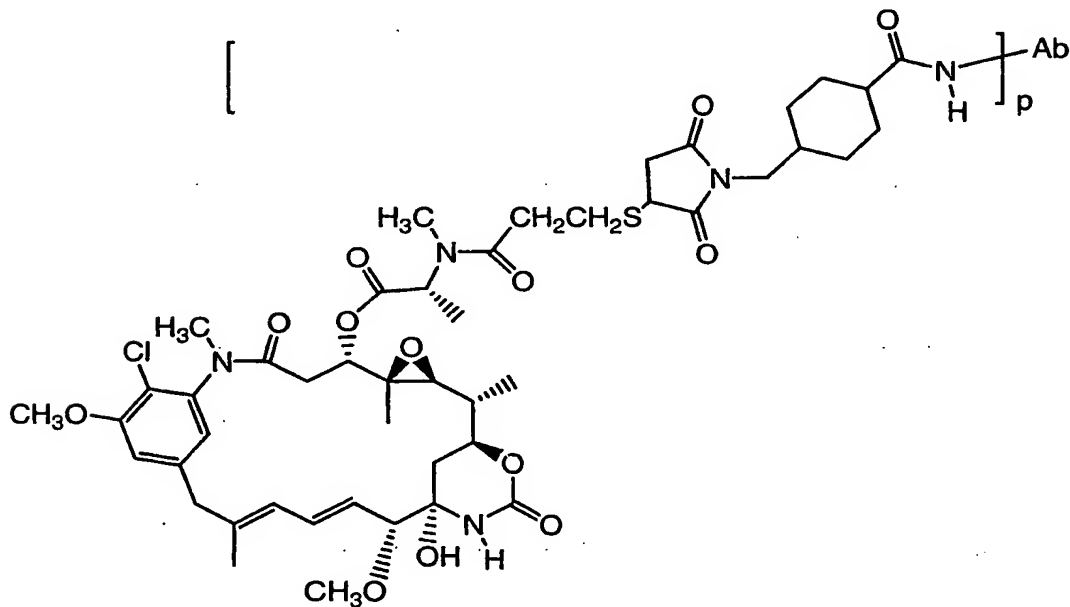


9. The antibody-drug conjugate compound of claim 3 having the structure:

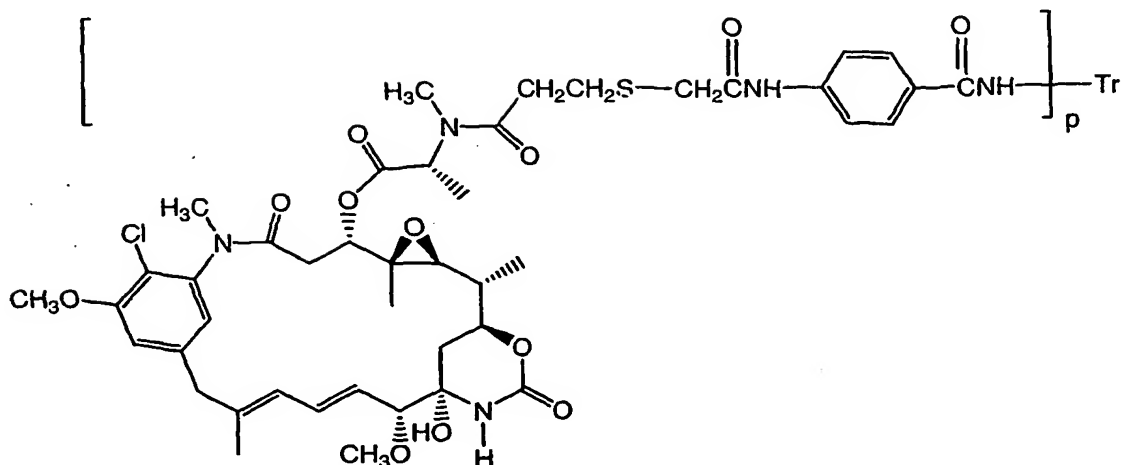


5

10. The antibody-drug conjugate compound of claim 1 having the structure:

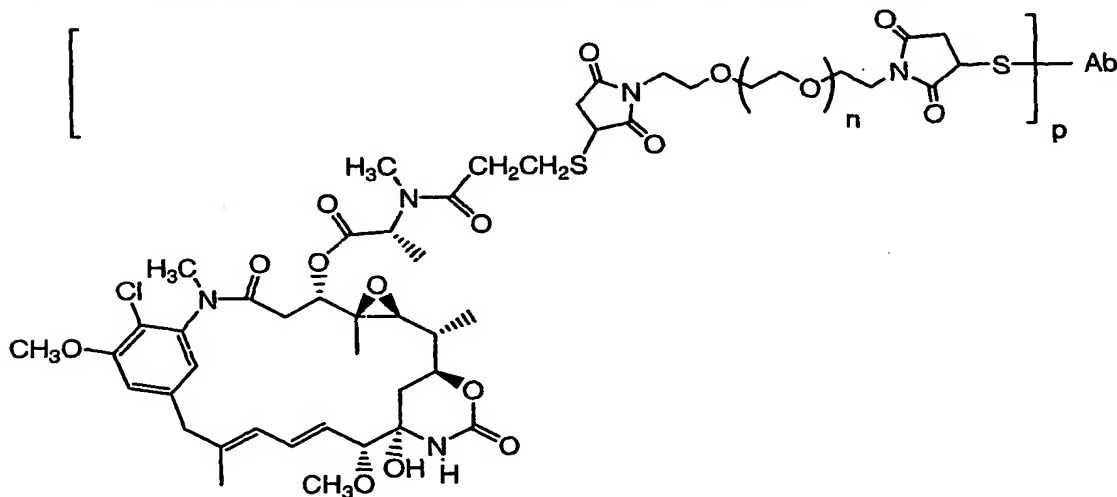


11. The antibody-drug conjugate of claim 10 wherein Ab is trastuzumab, and p is 1, 2, 3, or 4.
12. The antibody-drug conjugate compound of claim 1 having the structure:



where Tr is trastuzumab, and p is 1, 2, 3, or 4.

13. The antibody-drug conjugate compound of claim 1 having the structure:



wherein n is 0, 1, or 2; and p is 1, 2, 3, or 4.

14. The antibody-drug conjugate compound of claim 13 wherein Ab is trastuzumab.

15. The antibody-drug conjugate compound of claim 1 wherein p is 1, 2, 3, or 4.

16. The antibody-drug conjugate compound of claim 1 wherein the antibody binds to a receptor encoded by an ErbB gene.

17. The antibody-drug conjugate compound of claim 16 wherein the receptor is selected from EGFR, HER2, HER3 and HER4.

18. The antibody-drug conjugate compound of claim 17 wherein the antibody specifically binds to a HER2 receptor.

19. The antibody-drug conjugate compound of claim 1 which specifically binds to the extracellular domain of the HER2 receptor and inhibits growth of tumor cells which overexpress HER2 receptor.

20. The antibody-drug conjugate compound of any of the preceding claims wherein the antibody is selected from a monoclonal antibody, an antibody fragment, a chimeric antibody, and a humanized antibody.

21. The antibody-drug conjugate compound of claim 1 wherein the antibody is a humanized antibody selected from huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 (trastuzumab).

22. The antibody-drug conjugate compound of claim 21 wherein the antibody is huMAb4D5-8 (trastuzumab).

23. The antibody-drug conjugate of claim 20 wherein the antibody is a Fab fragment.

24. The antibody-drug conjugate compound of claim 1 wherein the antibody is attached to the linker through a cysteine thiol of the antibody.

25. The antibody-drug conjugate compound of claim 24 wherein p is 1, 2, 3, or 4.

26. A pharmaceutical composition comprising the antibody-drug conjugate compound of claim 1 or claim 2, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable diluent, carrier or excipient.

27. The pharmaceutical composition of claim 26 further comprising a therapeutically effective amount of a chemotherapeutic agent selected from Erlotinib, Bortezomib, Fulvestrant, Sutent, Letrozole, Imatinib mesylate, PTK787/ZK 222584, Oxaliplatin, 5-FU, Leucovorin, Rapamycin, Lapatinib, Lonaferib, Sorafenib, and Gefitinib.

28. The pharmaceutical composition of claim 26 further comprising a therapeutically effective amount of an anti-angiogenic agent.

29. The pharmaceutical composition of claim 26 further comprising a therapeutically effective amount of bevacizumab.

30. A method of inhibiting cellular proliferation comprising treating mammalian cells in a cell culture medium with an antibody-drug conjugate compound of claim 1 or claim 2, whereby proliferation of the cells is inhibited.

31. The method of claim 30 wherein the mammalian cells have HER2 receptor proteins to the antibody-drug conjugate compound.

32. The method of claim 31 wherein the mammalian cells are breast tumor cells.

33. The method of claim 30 wherein the antibody-drug conjugate compound is more cytotoxic than a maytansinoid compound comprising the maytansinoid moiety of the antibody-drug conjugate compound.

34. The method of claim 30 wherein the antibody-drug conjugate induces apoptosis.

5 35. A method of treating cancer comprising administering to a patient a formulation of an antibody-drug conjugate compound of claim 1 or claim 2, and a pharmaceutically acceptable diluent, carrier or excipient.

36. The method of claim 35 wherein the cancer is selected from the group consisting of breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, colorectal, thyroid, pancreatic, prostate and bladder cancer.

10 37. The method of claim 36 wherein the cancer is breast cancer which overexpresses ErbB2 at a 2+ level or more.

38. The method of claim 35 wherein the amount of antibody-drug conjugate compound administered to the patient is in the range of about 0.1 to about 10 mg/kg of patient weight per dose.

15 39. The method of claim 35 wherein the antibody-drug conjugate is administered at about three week intervals.

40. The method of claim 35 wherein the antibody-drug conjugate is administered by infusion.

41. The method of claim 35 wherein the antibody-drug conjugate is formulated with a pharmaceutically acceptable parenteral vehicle.

20 42. The method of claim 41 wherein the antibody-drug conjugate is formulated in a unit dosage injectable form.

43. The method of claim 42 wherein the antibody-drug conjugate is administered intravenously.

44. The method of claim 35 wherein the patient is administered a growth inhibitory antibody, in combination with the antibody-drug conjugate compound.

25 45. The method of claim 35 wherein the patient is administered a second antibody which binds ErbB2 and blocks ligand activation of an ErbB receptor, in combination with the antibody-drug conjugate compound.

46. The method of claim 45 wherein the second antibody comprises monoclonal antibody 2C4 or humanized 2C4.

47. The method of claim 46 wherein said second antibody is conjugated with a cytotoxic agent.

48. The method of claim 35 wherein the patient is administered a chemotherapeutic agent, in combination with the antibody-drug conjugate compound, where the chemotherapeutic agent is selected from Erlotinib, Bortezomib, Fulvestrant, Sutent, Letrozole, Imatinib mesylate, PTK787/ZK 222584, Oxaliplatin, 5-FU, Leucovorin, Rapamycin, Lapatinib, Lonaframib, Sorafenib, and Gefitinib.

5 49. The method of claim 35 wherein the patient is administered an anti-angiogenic agent in combination with the antibody-drug conjugate compound where the anti-angiogenic agent is selected from bevacizumab.

10 50. A method of inhibiting the growth of tumor cells that overexpress a growth factor receptor, comprising administering to a patient an antibody-drug conjugate of claim 1 which binds specifically to a growth factor receptor selected from HER2 or EGF and a chemotherapeutic agent wherein said antibody-drug conjugate and said chemotherapeutic agent are each administered in amounts effective to inhibit growth of tumor cells in the patient.

15 51. A method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor, comprising administering a combination of an antibody-drug conjugate of claim 1 and a chemotherapeutic agent or a growth inhibitory agent.

52. The method of claim 51 wherein the antibody of the antibody-drug conjugate is an anti-ErbB2 antibody.

53. The method of claim 52 wherein the anti-ErbB2 antibody has a biological characteristic of a 4D5 monoclonal antibody.

20 54. The method of claim 52 wherein the anti-ErbB2 antibody binds essentially the same epitope as a 4D5 monoclonal antibody.

55. The method of claim 52 wherein the anti-ErbB2 antibody is selected from the group consisting of humanized antibodies huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 (trastuzumab).

25 56. An assay for detecting cancer cells comprising:  
(a) exposing cells to an antibody-drug conjugate compound of claim 1 or claim 2; and  
(b) determining the extent of binding of the antibody-drug conjugate compound to the cells.

57. The assay of claim 56 wherein the cells are breast tumor cells.

30 58. The assay of claim 56 wherein the extent of binding is determined by measuring levels of ErbB-encoding nucleic acid by fluorescent in situ hybridization (FISH).

59. The assay of claim 56 wherein the extent of binding is determined by immunohistochemistry (IHC).

60. An article of manufacture comprising  
 an antibody-drug conjugate compound of claim 1 or claim 2;  
 a container; and  
 a package insert or label indicating that the compound can be used to treat cancer characterized by the  
 5 overexpression of an ErbB receptor.

61. The article of manufacture of claim 60 wherein said package insert of label indicates that the  
 compound can be used to treat cancer characterized by the overexpression of an ErbB2 receptor.

62. The article of manufacture of claim 61 wherein the cancer is breast cancer.

63. The article of manufacture of claim 62 wherein the cancer is characterized by the  
 10 overexpression of an ErbB2 receptor at a 2+ level or above.

64. A method of making an antibody-drug conjugate compound comprising an antibody  
 covalently attached by a linker to one or more maytansinoid drug moieties, the compound having Formula I:



or a pharmaceutically acceptable salt or solvate thereof, wherein:

15 Ab is an antibody which binds to an ErbB receptor, or which binds to one or more tumor-associated  
 antigens or cell-surface receptors selected from (1)-(36):

(1) BMPR1B (bone morphogenetic protein receptor-type IB, Genbank accession no. NM\_001203);

(2) E16 (LAT1, SLC7A5, Genbank accession no. NM\_003486);

(3) STEAP1 (six transmembrane epithelial antigen of prostate, Genbank accession no. NM\_012449);

20 (4) 0772P (CA125, MUC16, Genbank accession no. AF361486);

(5) MPF (MPF, MSLN, SMR, megakaryocyte potentiating factor, mesothelin, Genbank accession no.  
 NM\_005823);

(6) Napi3b (NAPI-3B, NPTIb, SLC34A2, solute carrier family 34 (sodium phosphate), member 2,  
 type II sodium-dependent phosphate transporter 3b, Genbank accession no. NM\_006424);

25 (7) Sema 5b (FLJ10372, KIAA1445, Mm.42015, SEMA5B, SEMAG, Semaphorin 5b Hlog, sema  
 domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short  
 cytoplasmic domain, (semaphorin) 5B, Genbank accession no. AB040878);

(8) PSCA hlg (2700050C12Rik, C530008O16Rik, RIKEN cDNA 2700050C12, RIKEN cDNA  
 2700050C12 gene, Genbank accession no. AY358628);

30 (9) ETBR (Endothelin type B receptor, Genbank accession no. AY275463);

(10) MSG783 (RNF124, hypothetical protein FLJ20315, Genbank accession no. NM\_017763);

(11) STEAP2 (HGNC\_8639, IPCA-1, PCANAP1, STAMP1, STEAP2, STMP, prostate cancer  
 associated gene 1, prostate cancer associated protein 1, six transmembrane epithelial antigen of prostate 2, six  
 transmembrane prostate protein, Genbank accession no. AF455138);

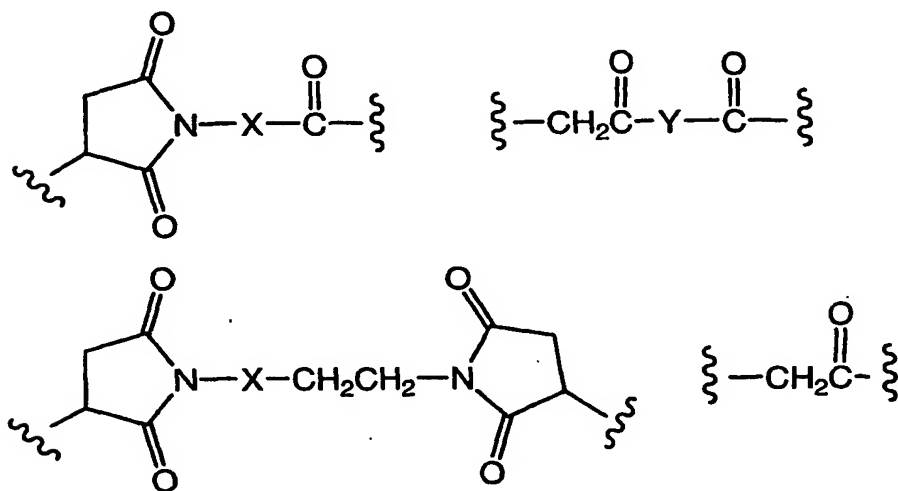
- (12) TrpM4 (BR22450, FLJ20041, TRPM4, TRPM4B, transient receptor potential cation channel, subfamily M, member 4, Genbank accession no. NM\_017636);
- (13) CRIPTO (CR, CR1, CRGF, CRIPTO, TDGF1, teratocarcinoma-derived growth factor, Genbank accession no. NP\_003203 or NM\_003212);
- 5 (14) CD21 (CR2 (Complement receptor 2) or C3DR (C3d/Epstein Barr virus receptor) or Hs.73792 Genbank accession no. M26004);
- (15) CD79b (CD79B, CD79 $\beta$ , Ig $\beta$  (immunoglobulin-associated beta), B29, Genbank accession no. NM\_000626);
- (16) FcRH2 (IFGP4, IRTA4, SPAP1A (SH2 domain containing phosphatase anchor protein 1a),  
10 SPAP1B, SPAP1C, Genbank accession no. NM\_030764);
- (17) HER2 (Genbank accession no. M11730);
- (18) NCA (Genbank accession no. M18728);
- (19) MDP (Genbank accession no. BC017023);
- (20) IL20R $\alpha$  (Genbank accession no. AF184971);
- 15 (21) Brevican (Genbank accession no. AF229053);
- (22) EphB2R (Genbank accession no. NM\_004442);
- (23) ASLG659 (Genbank accession no. AX092328);
- (24) PSCA (Genbank accession no. AJ297436);
- (25) GEDA (Genbank accession no. AY260763);
- 20 (26) BAFF-R (B cell -activating factor receptor, BLyS receptor 3, BR3, NP\_443177.1);
- (27) CD22 (B-cell receptor CD22-B isoform, NP-001762.1);
- (28) CD79a (CD79A, CD79 $\alpha$ , immunoglobulin-associated alpha, a B cell-specific protein that covalently interacts with Ig beta (CD79B) and forms a complex on the surface with Ig M molecules, transduces a signal involved in B-cell differentiation, Genbank accession No. NP\_001774.1);
- 25 (29) CXCR5 (Burkitt's lymphoma receptor 1, a G protein-coupled receptor that is activated by the CXCL13 chemokine, functions in lymphocyte migration and humoral defense, plays a role in HIV-2 infection and perhaps development of AIDS, lymphoma, myeloma, and leukemia, Genbank accession No. NP\_001707.1);
- (30) HLA-DOB (Beta subunit of MHC class II molecule (Ia antigen) that binds peptides and presents  
30 them to CD4+ T lymphocytes, Genbank accession No. NP\_002111.1);
- (31) P2X5 (Purinergic receptor P2X ligand-gated ion channel 5, an ion channel gated by extracellular ATP, may be involved in synaptic transmission and neurogenesis, deficiency may contribute to the pathophysiology of idiopathic detrusor instability, Genbank accession No. NP\_002552.2);
- (32) CD72 (B-cell differentiation antigen CD72, Lyb-2, Genbank accession No. NP\_001773.1);
- 35 (33) LY64 (Lymphocyte antigen 64 (RP105), type I membrane protein of the leucine rich repeat (LRR) family, regulates B-cell activation and apoptosis, loss of function is associated with increased disease activity in patients with systemic lupus erythematosus, Genbank accession No. NP\_005573.1);
- (34) FcRH1 (Fc receptor-like protein 1, a putative receptor for the immunoglobulin Fc domain that  
40 contains C2 type Ig-like and ITAM domains, may have a role in B-lymphocyte differentiation, Genbank accession No. NP\_443170.1);

(35) IRTA2 (Immunoglobulin superfamily receptor translocation associated 2, a putative immunoreceptor with possible roles in B cell development and lymphomagenesis; deregulation of the gene by translocation occurs in some B cell malignancies, Genbank accession No. NP\_112571.1); and

(36) TENB2 (putative transmembrane proteoglycan, related to the EGF/herregulin family of growth factors and follistatin, Genbank accession No. AF179274;

provided that the antibody is not TA.1;

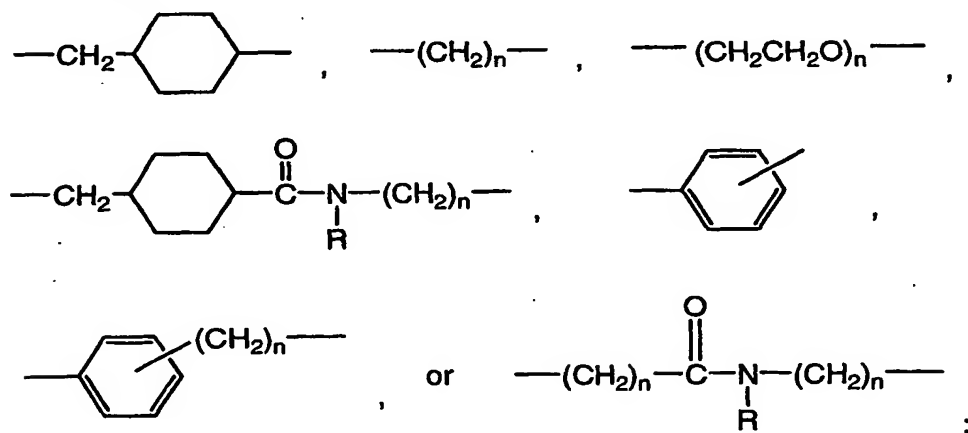
L is a linker selected from the structures;



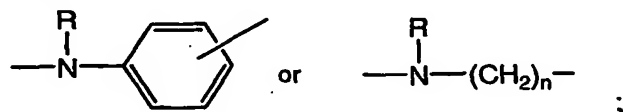
where the wavy lines indicates the covalent attachments to Ab and D;

10

X is:



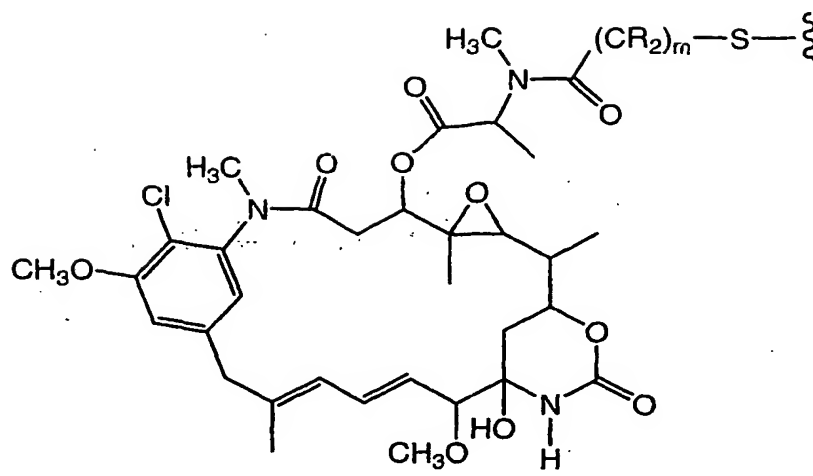
Y is:



where R is independently H or C<sub>1</sub>-C<sub>6</sub> alkyl; and n is 1 to 12;

15

D is a maytansinoid drug moiety selected from the structure:



where the wavy line indicates the covalent attachment to L; R is independently H or C<sub>1</sub>-C<sub>6</sub> alkyl; m is 1, 2, or 3; and p is 1 to 8;

wherein the method comprises:

- 5        reacting Ab with a linker reagent to form antibody-linker intermediate Ab-L, and then reacting Ab-L with a drug moiety D to form the antibody-drug conjugate; or
- reacting a drug moiety D with a linker reagent to form a drug-linker intermediate D-L, and then reacting D-L with Ab to form the antibody-drug conjugate.

- 10       65.        The method of claim 64 wherein Ab is an antibody which binds to one or more tumor-associated antigens or cell-surface receptors selected from (1)-(16) and (18)-(36).

66.        The method of claim 64 or claim 65 wherein the linker reagent is SMCC.

67.        The method of claim 64 or claim 65 wherein the linker reagent is a bis-maleimide reagent selected from DTME, BMB, BMDB, BMH, BMOE, BM(PEO)<sub>3</sub>, and BM(PEO)<sub>4</sub>.

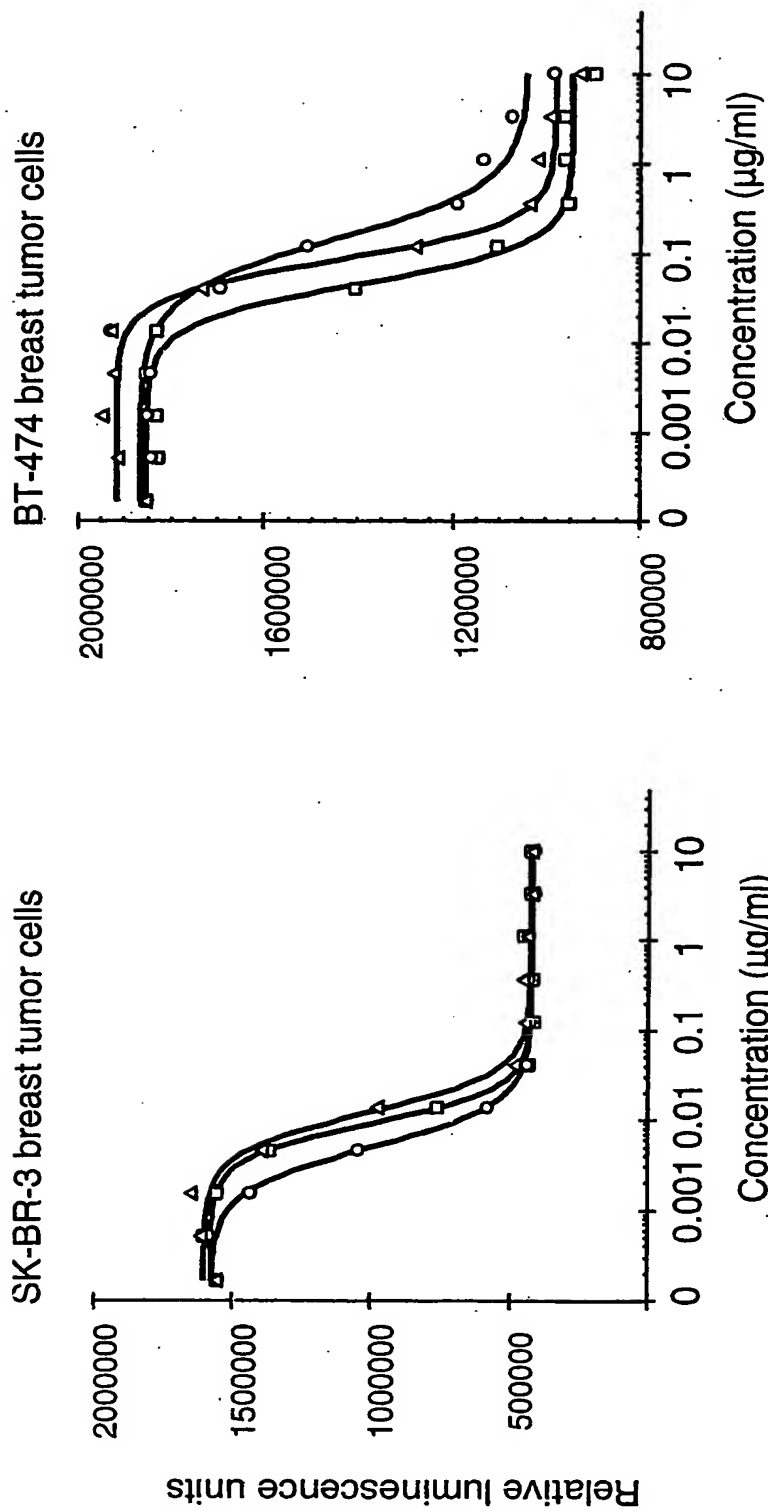


Figure 2

Figure 1

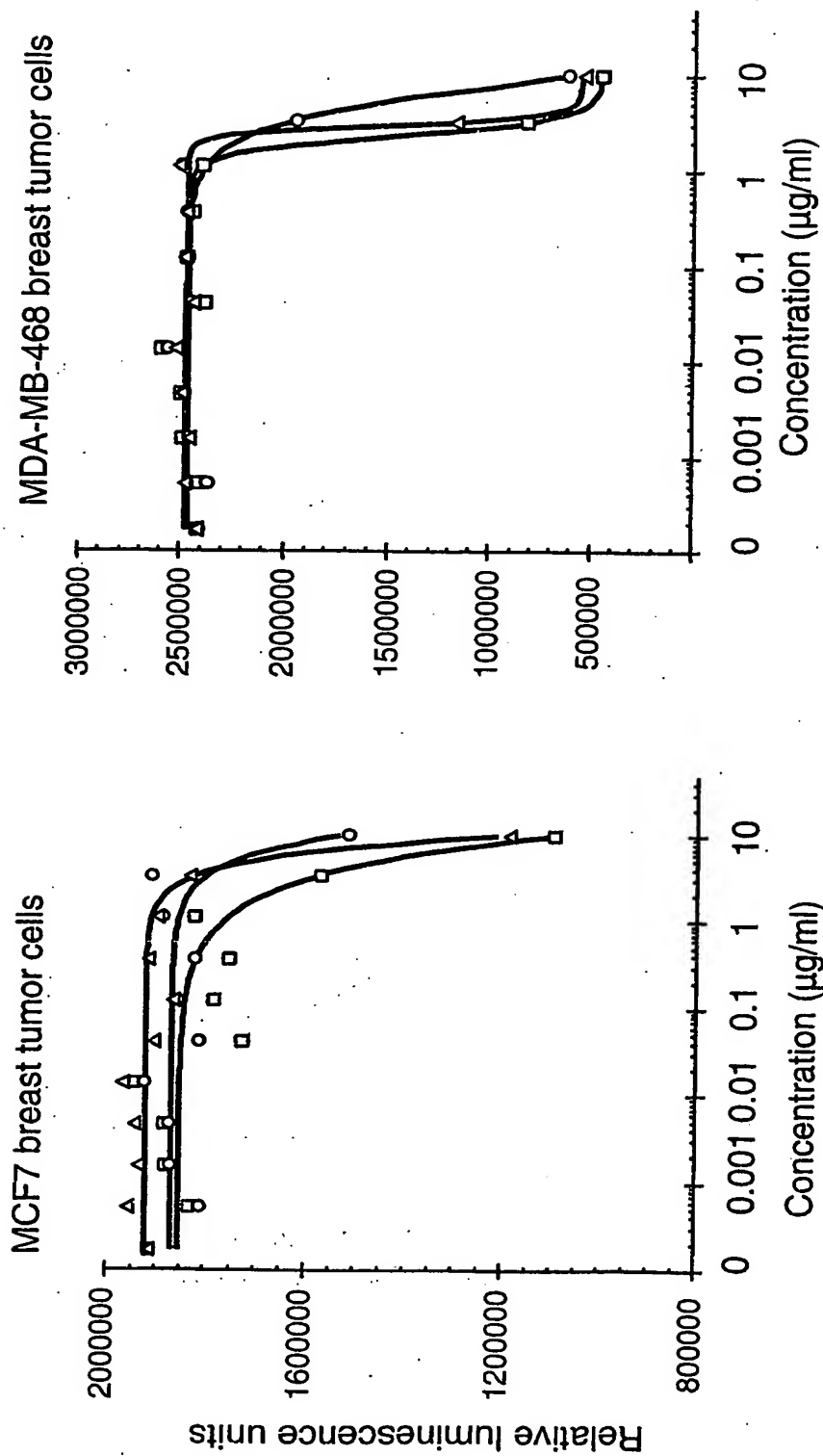


Figure 4

Figure 3

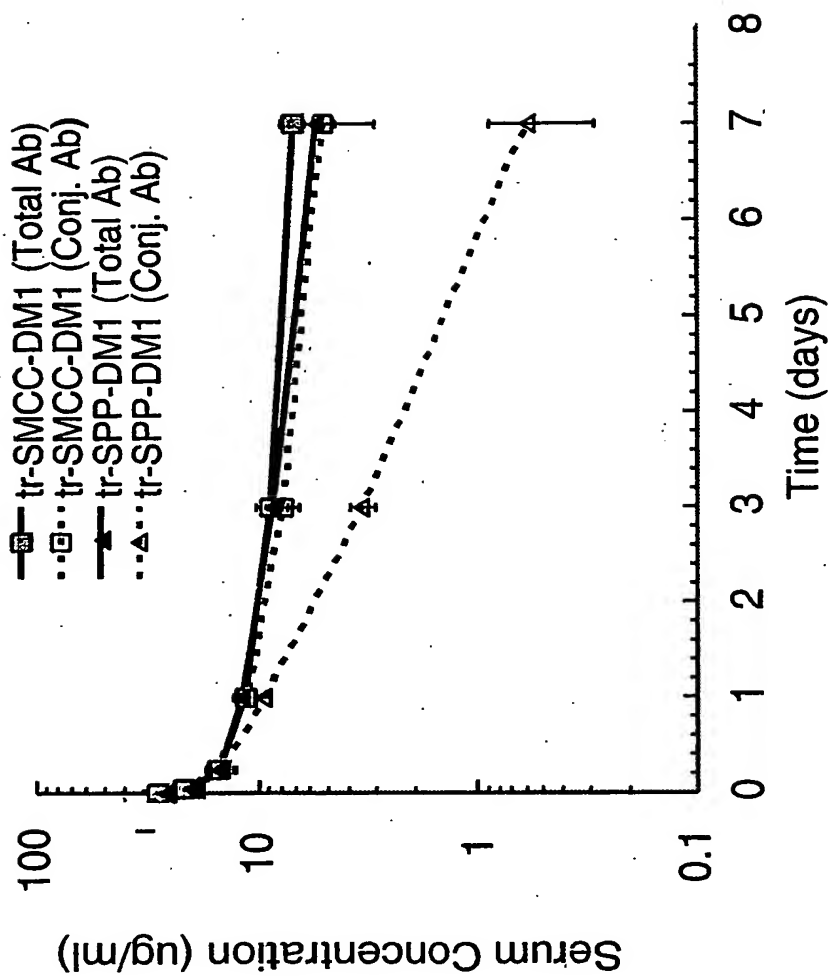


Figure 5

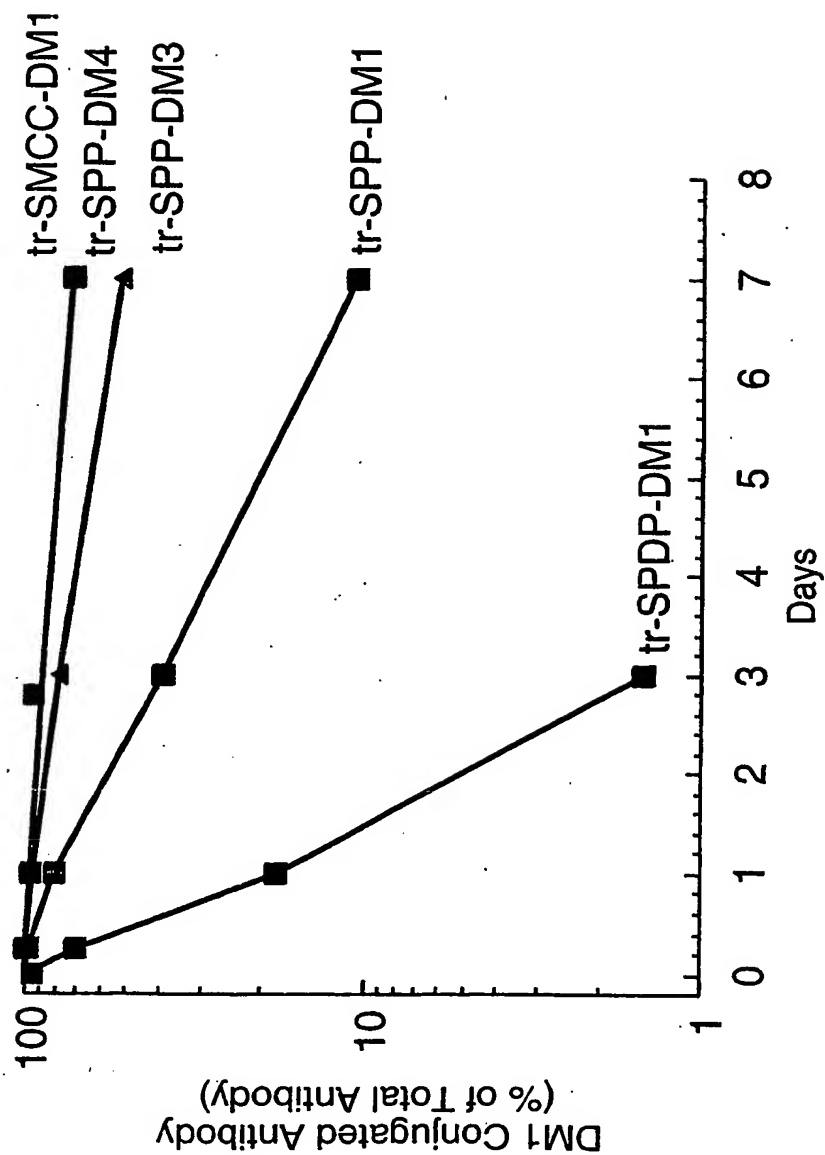


Figure 6

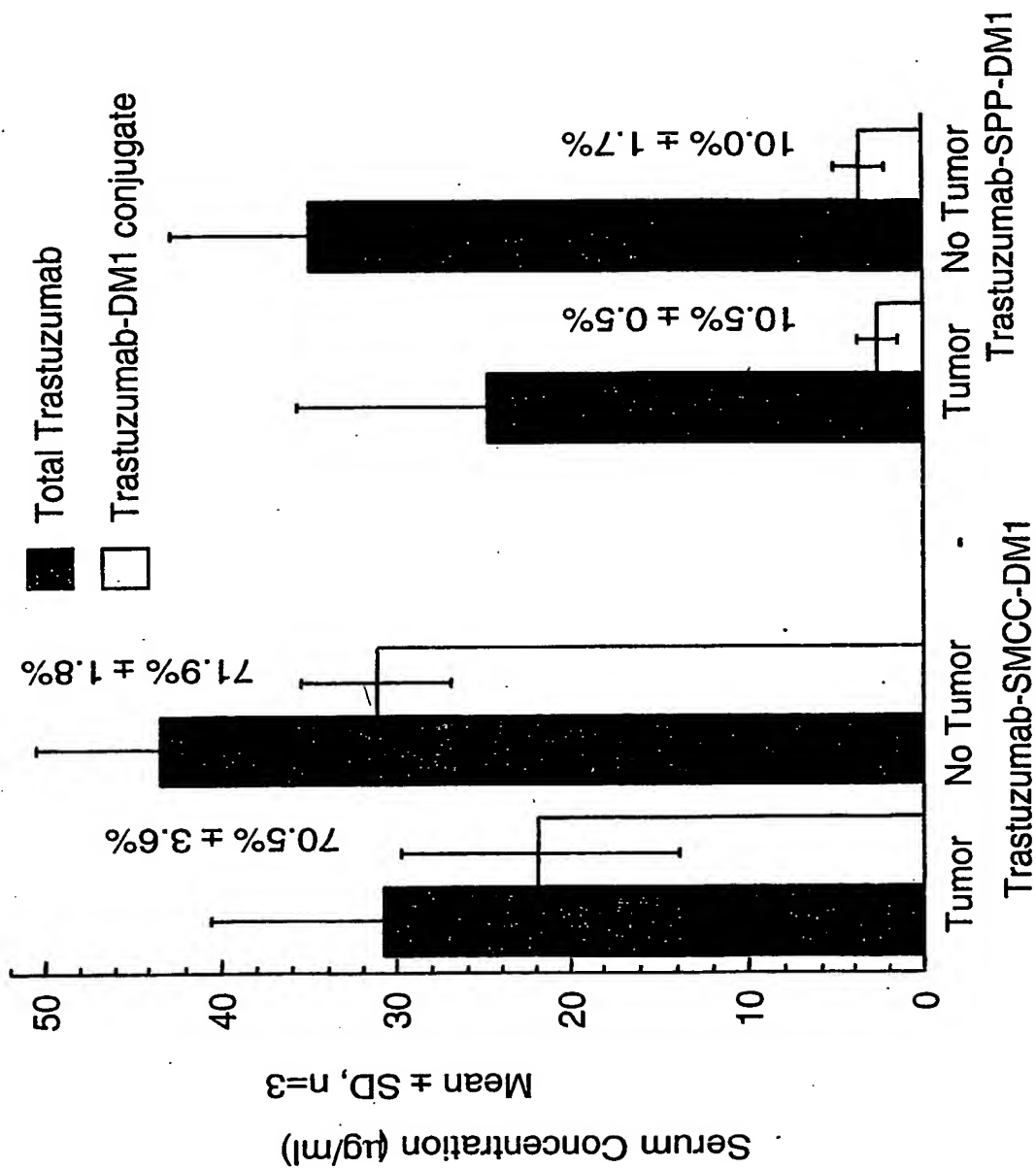


Figure 7

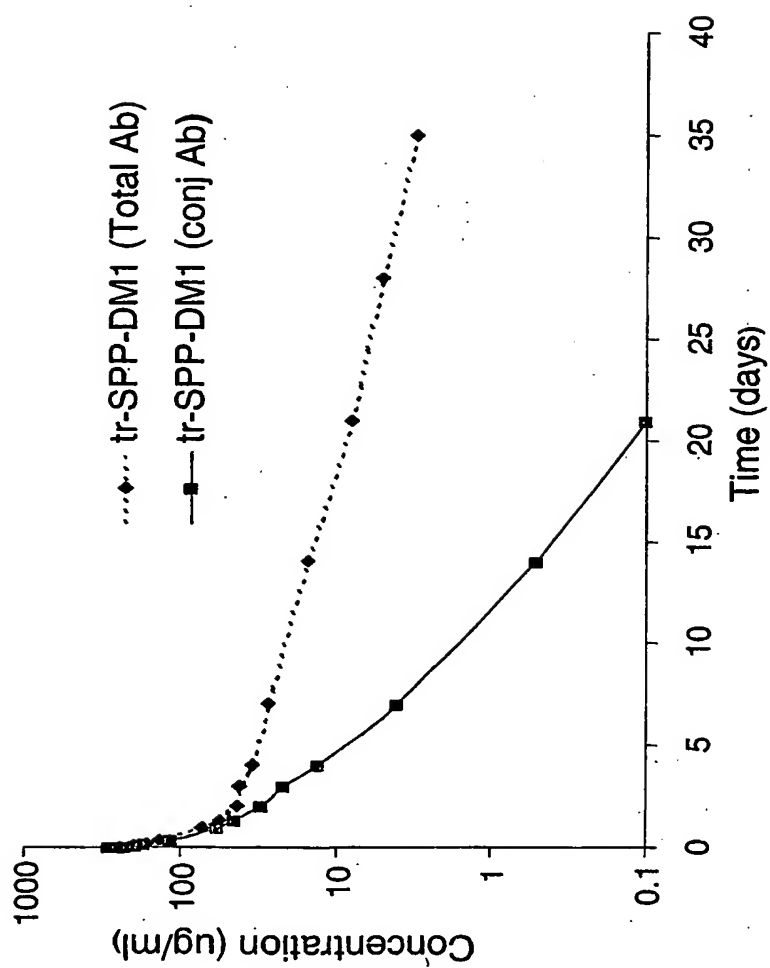


Figure 8

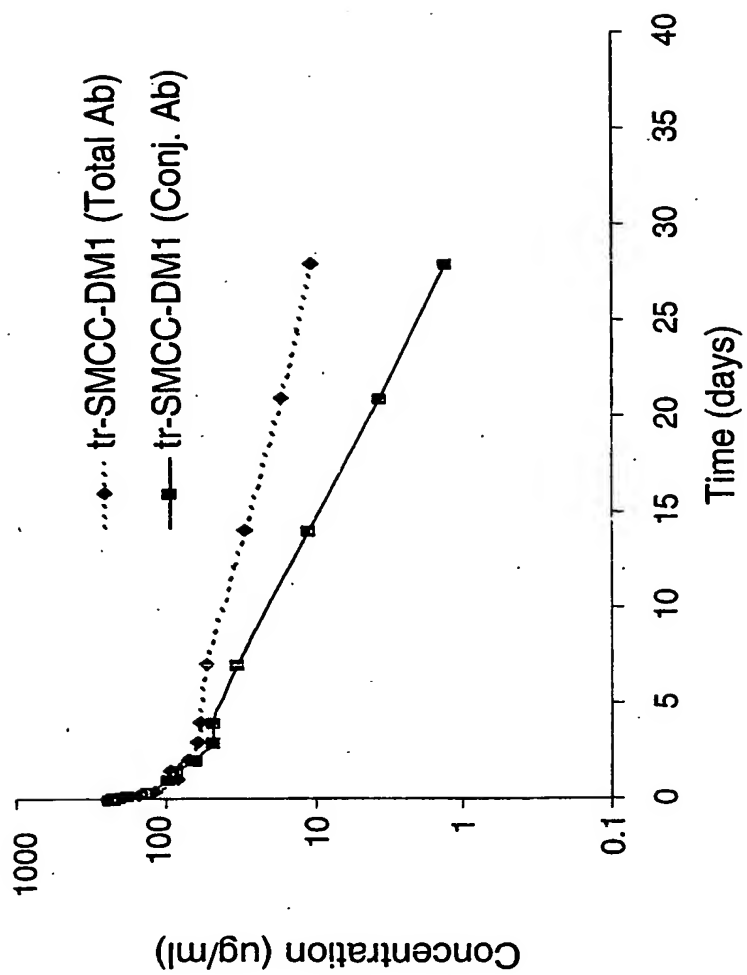


Figure 9

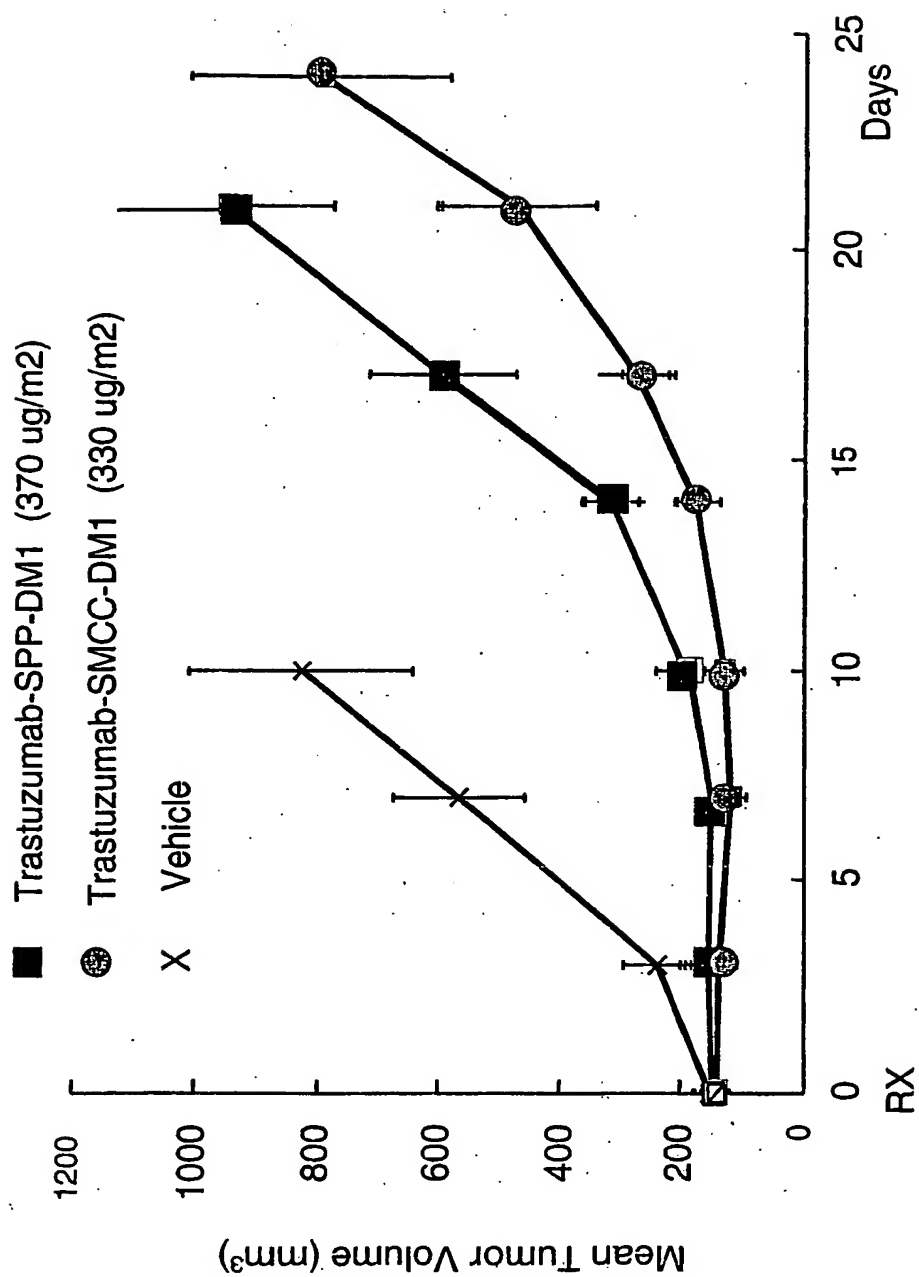


Figure 10

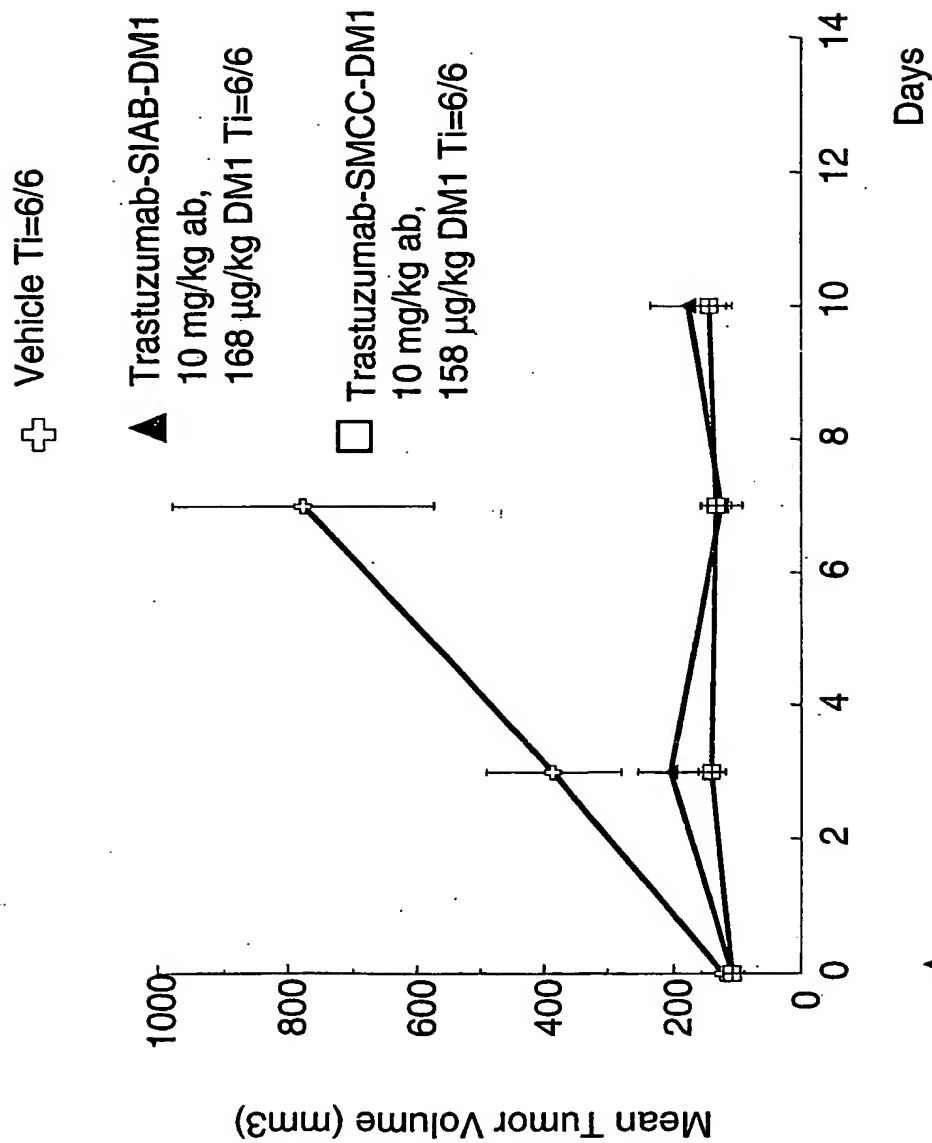


Figure 11

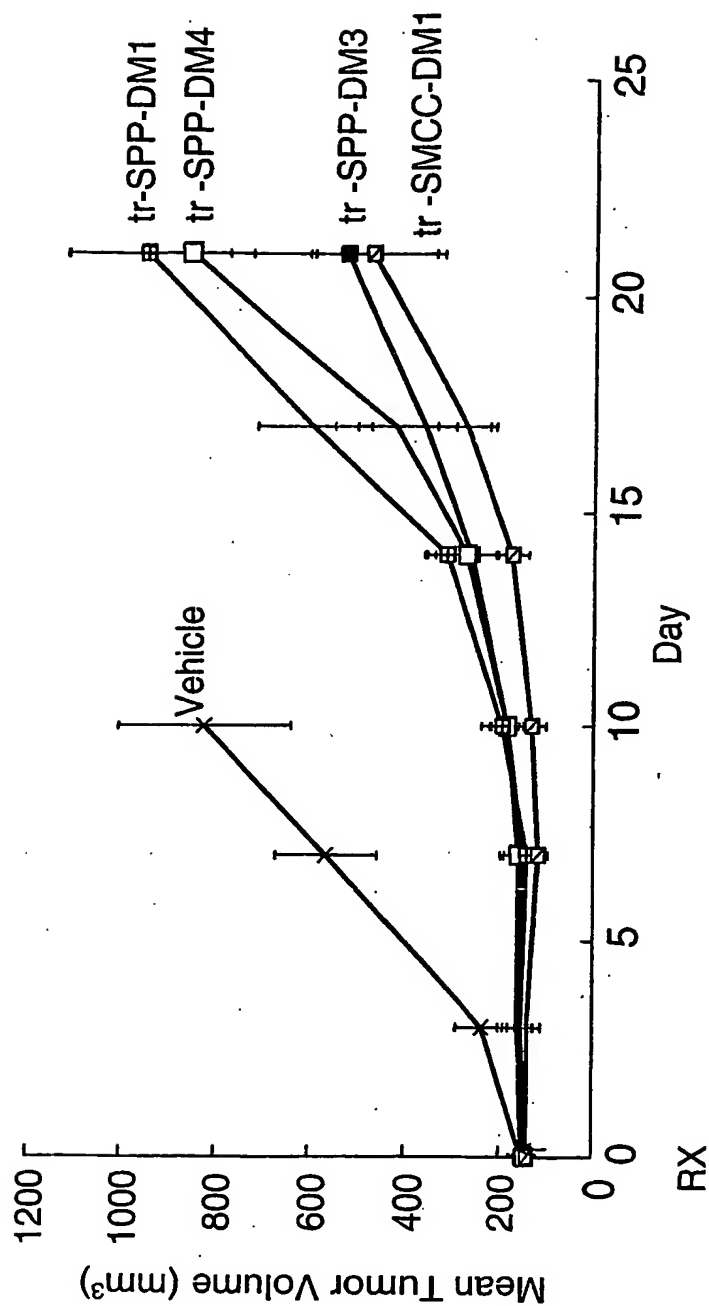
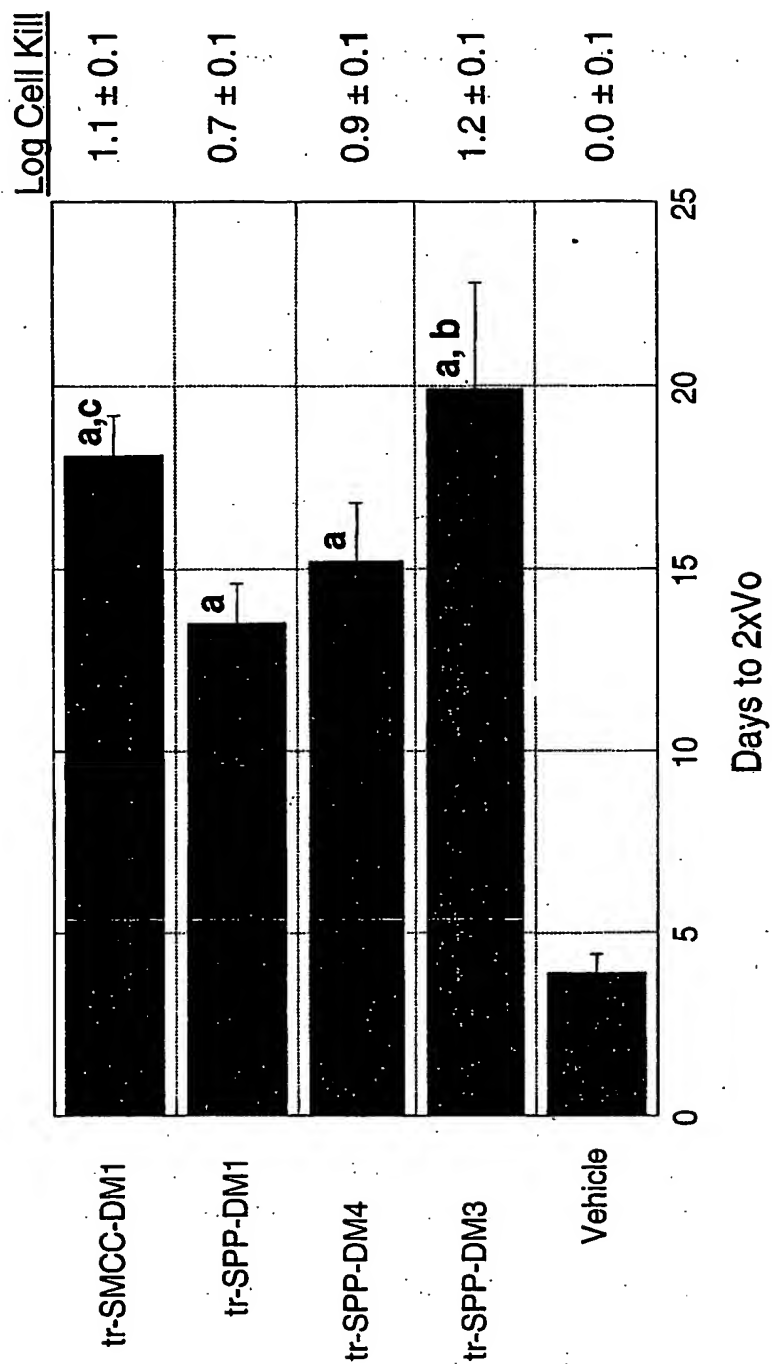


Figure 12

## Trastuzumab-Conjugates in HER2-Fo5



a P<0.0003 vs Vehicle      b P=0.010 vs tr-SPP-DM1      c P=0.61 vs tr-SPP-DM1

Figure 13

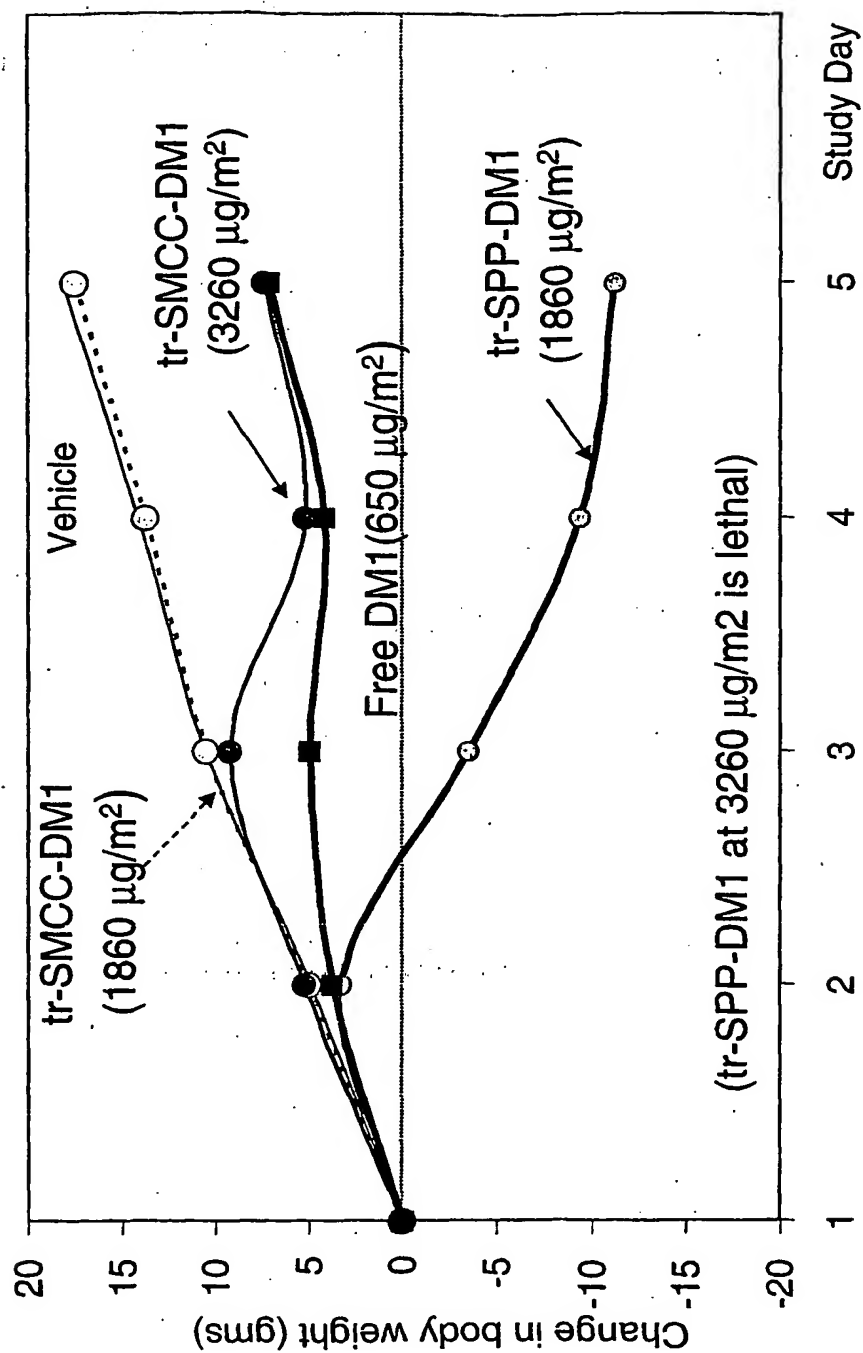
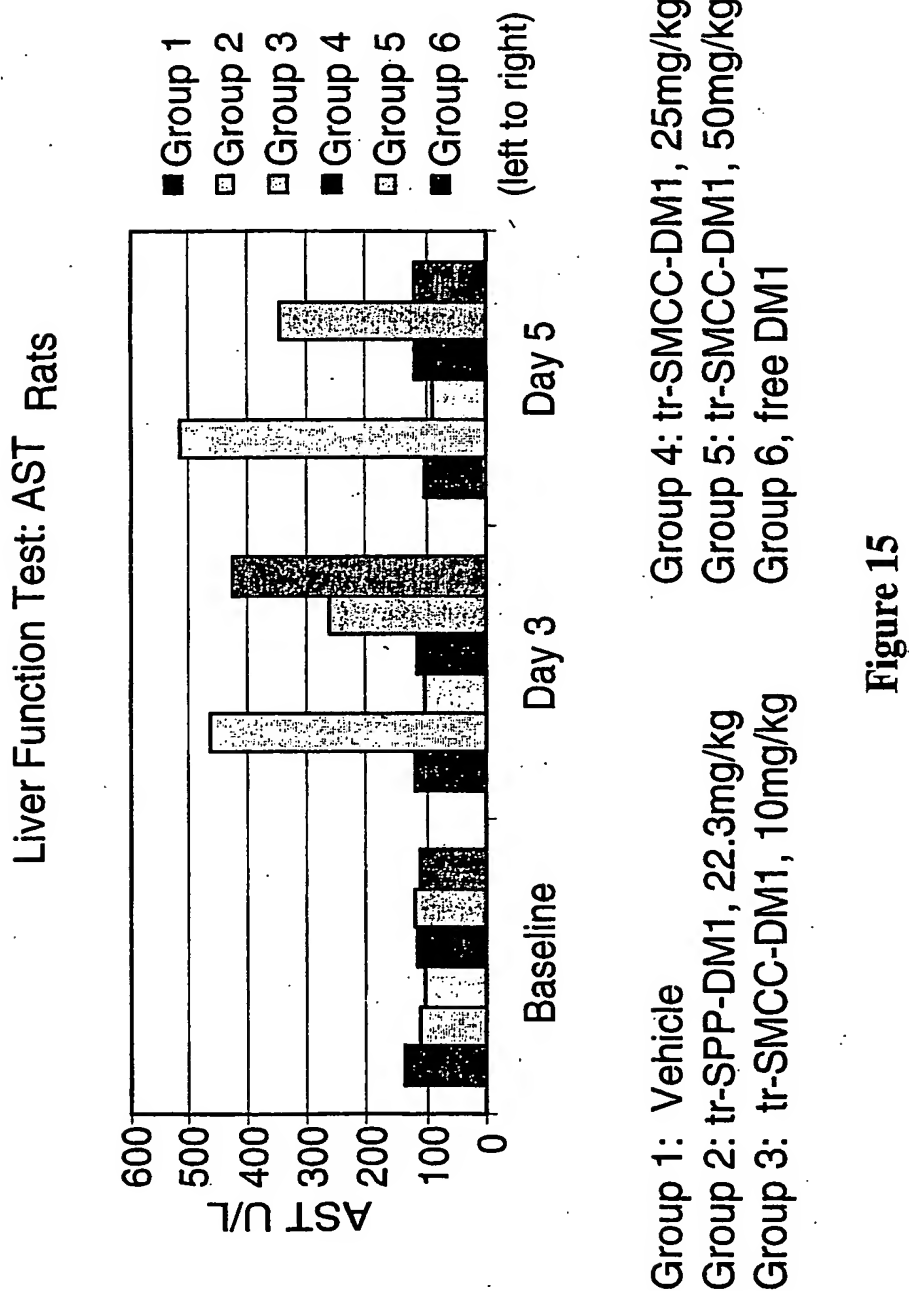


Figure 14



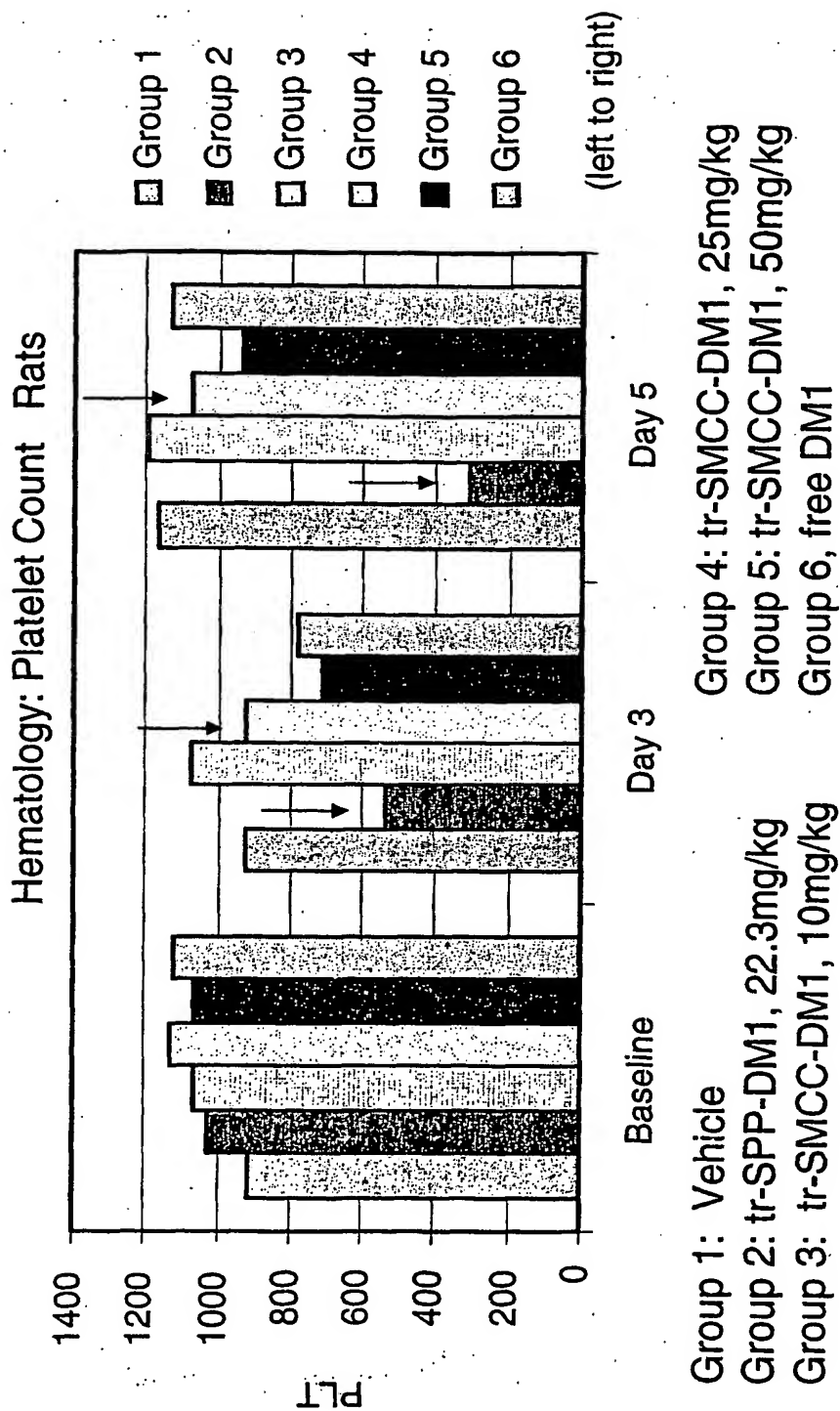


Figure 16

Group 1: Vehicle  
 Group 2: tr-SPP-DM1, 22.3mg/kg  
 Group 3: tr-SMCC-DM1, 10mg/kg  
 Group 4: tr-SMCC-DM1, 25mg/kg  
 Group 5: tr-SMCC-DM1, 50mg/kg  
 Group 6: free DM1

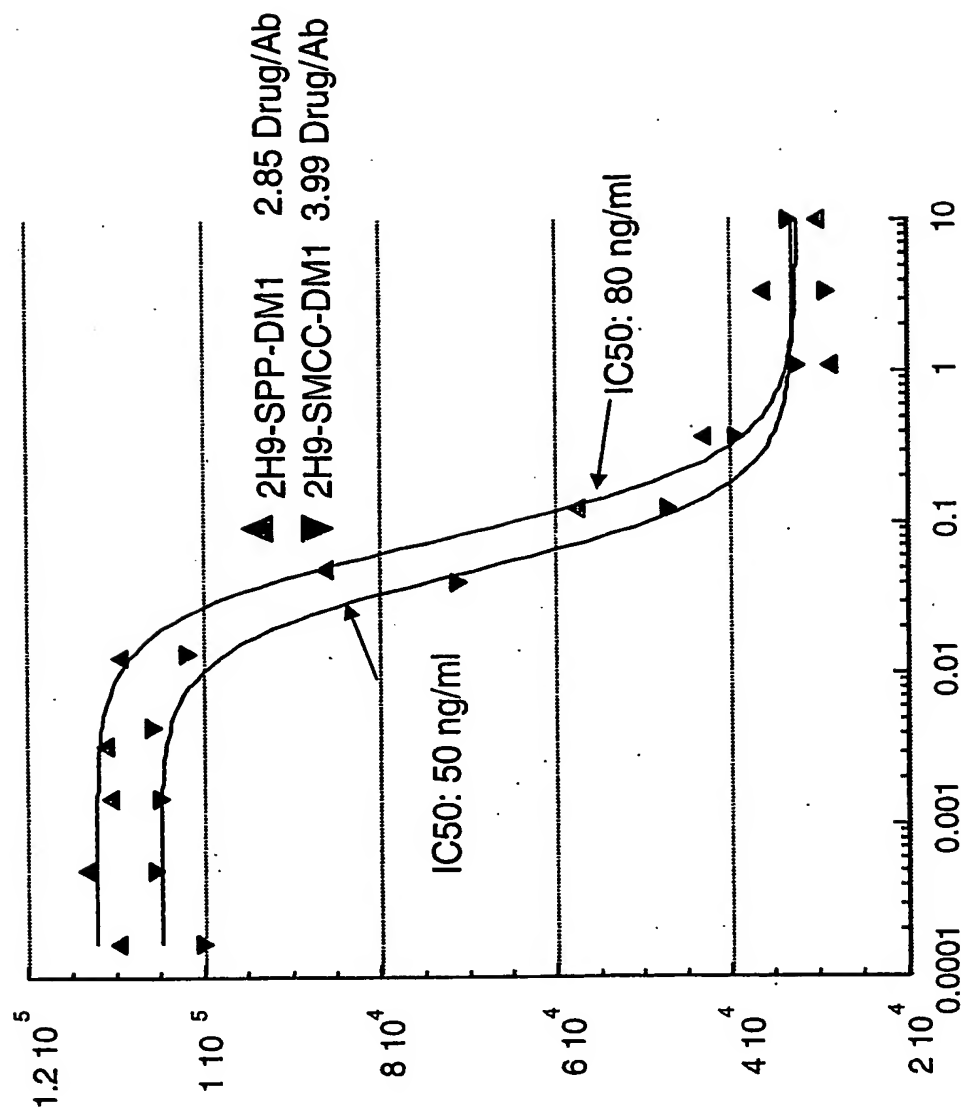


Figure 17

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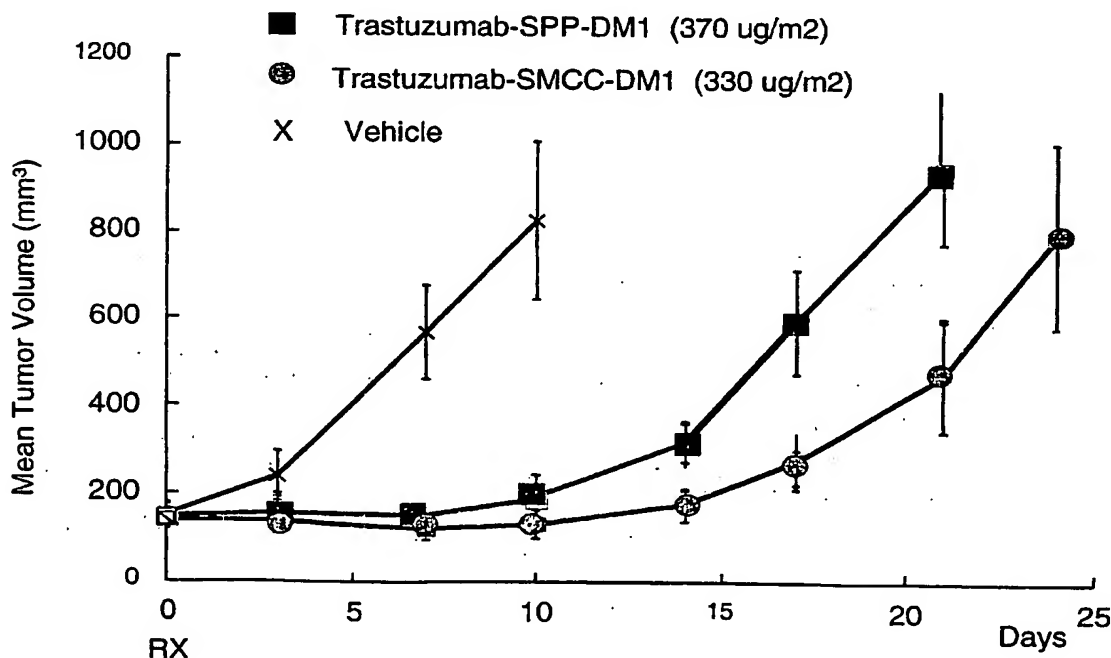
(74) Agent: ANDRUS, Alex; Genentech, Inc., MS 49, 1 DNA Way, South San Francisco, CA 94080 (US).

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[Continued on next page]

(54) Title: ANTIBODY DRUG CONJUGATES AND METHODS



(57) Abstract: The present invention relates to antibody-drug conjugate compounds of Formula I: Ab-(L-D)<sub>p</sub> where one or more maytansinoid drug moieties (D) are covalently linked by L to an antibody (Ab) which binds to an ErbB receptor, or which binds to one or more tumor-associated antigens or cell-surface receptors. These compounds may be used in methods of diagnosis or treatment of cancer, and other diseases and disorders.

WO 2005/117986 A3



SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(88) Date of publication of the international search report:  
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# INTERNATIONAL SEARCH REPORT

International application No

PCT/US2005/018829

**A. CLASSIFICATION OF SUBJECT MATTER**  
A61K47/48 A61P35/00 G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, MEDLINE, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 01/00244 A (GENENTECH, INC; ERICKSON, SHARON; SCHWALL, RALPH; SLIWKOWSKI, MARK, X) 4 January 2001 (2001-01-04)</p> <p>page 37, line 5 - line 30; claims 17,18,25,28,29,31,32,37-41; figure 1</p> <p>page 10, line 10 - line 13</p> <p>page 14, line 11 - line 27</p> <p>page 38, line 13 - line 19</p> <p>page 38, line 35 - page 43, line 3</p> <p style="text-align: center;">-/--</p>	<p>1,3-8, 10,11, 15-64,66</p>

☒ Further documents are listed in the continuation of Box C.

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Date of the actual completion of the international search

19 January 2006

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PCT/US2005/018829

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>CHARI R V J ET AL: "IMMUNOCONJUGATES CONTAINING NOVEL MAYTANSINOIDS: PROMISING ANTICANCER DRUGS"  CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, US,  vol. 52, no. 1, January 1992 (1992-01),  pages 127-131, XP000453560  ISSN: 0008-5472  page 127, right-hand column, paragraph 3;  figure 2  page 128, right-hand column, last  paragraph - page 129, left-hand column,  paragraph 3</p>	1-8,10, 15,20, 23,25, 64-66
Y	<p>EP 0 425 235 A (IMMUNOGEN INC)  2 May 1991 (1991-05-02)</p> <p>page 10, line 52 - line 55  page 9, line 5 - line 42; examples 3,4</p>	1-8,10, 15,20, 23,25, 64-66
Y	<p>WO 01/24763 A (IMMUNOGEN, INC; CHARI,  RAVI, V., J) 12 April 2001 (2001-04-12)</p> <p>page 21, line 13 - page 22, line 2  page 17, line 3 - page 19, line 3</p>	1-8,10, 15,20, 23,25, 64-66
A	<p>TRAIL P A ET AL: "EFFECT OF LINKER  VARIATION ON THE STABILITY, POTENCY, AND  EFFICACY OF CARCINOMAREACTIVE  BR64-DOXORUBICIN IMMUNOCONJUGATES"  CANCER RESEARCH, AMERICAN ASSOCIATION FOR  CANCER RESEARCH, BALTIMORE, MD, US,  vol. 57, 1 January 1997 (1997-01-01),  pages 100-105, XP001019049  ISSN: 0008-5472  the whole document</p>	
P,X	<p>WO 2005/037992 A (IMMUNOGEN, INC; STEEVES,  RITA; LUTZ, ROBERT; CHARI, RAVI; XIE,  HONGSHE) 28 April 2005 (2005-04-28)  cited in the application  page 12, paragraph '64!', page 20,  paragraph '94!', page 23 paragraph '112!  to page 25 paragraph '116!' and page 30  paragraph '141!' to page 31 paragraph '143!  figures 3,4,6a-33,35,36; examples 1a-4</p> <p style="text-align: center;">-/--</p>	1,3-8, 10,11, 15-64,66

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/US2005/018829

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 2004/016225 A (GENENTECH, INC) 26 February 2004 (2004-02-26) cited in the application</p> <p>page 82, line 5 - line 35; figures 96,140 page 104, line 3 - page 109, line 26</p>	<p>1-10,13, 15,20, 23-30, 33-49, 56-67</p>
X	<p>WO 03/000113 A (GENENTECH, INC) 3 January 2003 (2003-01-03)</p> <p>page 103, line 19 - page 109, line 6 page 81, line 31 - page 83, line 2 page 5, line 23 - line 26; figures 51,129</p>	<p>1-10,13, 15,20, 23-30, 33-49, 56-67</p>
X	<p>WO 03/024392 A (GENENTECH, INC) 27 March 2003 (2003-03-27)</p> <p>page 100, line 31 - page 106, line 11 page 79, line 29 - page 80, line 21; figure 97</p>	<p>1-10,13, 15,20, 23-30, 33-49, 56-67</p>
X	<p>WO 02/16429 A (GENENTECH, INC; GODDARD, AUDREY; GODOWSKI, PAUL, J; GURNEY, AUSTIN, L;) 28 February 2002 (2002-02-28)</p> <p>page 5, line 27 - line 30; figure 9 page 52, line 24 - page 53, line 32 page 71, line 33 - page 77, line 3</p>	<p>1-10,13, 15,20, 23-30, 33-49, 56-67</p>
Y	<p>WO 03/022995 A (AGENSYS, INC) 20 March 2003 (2003-03-20)</p> <p>page 35, paragraph 5 - page 37, paragraph 4 page 50, paragraph 2 - page 51, paragraph 4</p>	<p>1-10,13, 15,20, 23-30, 33-49, 56-67</p>
X	<p>WO 2004/005470 A (IMMUNOGEN, INC) 15 January 2004 (2004-01-15)</p>	<p>1,2</p>
Y	<p>abstract; example 14</p> <p>page 5, paragraph 3 page 25, paragraph 2 - paragraph 3</p>	<p>3-10,13, 15,20, 23-30, 33-49, 56-67</p>
	-/--	

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HU XIU FENG ET AL: "Discovery and validation of new molecular targets for ovarian cancer." CURRENT OPINION IN MOLECULAR THERAPEUTICS. DEC 2003, vol. 5, no. 6, December 2003 (2003-12), pages 625-630, XP009059821 ISSN: 1464-8431 page 627, right-hand column, paragraph 2 - page 628, left-hand column, paragraph 3 -----	1-10,13, 15,20, 23-30, 33-49, 56-67
A	CHU P G ET AL: "CD79: a review." APPLIED IMMUNOHISTOCHEMISTRY & MOLECULAR MORPHOLOGY : AIMM / OFFICIAL PUBLICATION OF THE SOCIETY FOR APPLIED IMMUNOHISTOCHEMISTRY. JUN 2001, vol. 9, no. 2, June 2001 (2001-06), pages 97-106, XP009059897 ISSN: 1541-2016 the whole document -----	
Y	WO 03/072036 A (DUKE UNIVERSITY; UNIVERSITY OF CALIFORNIA; TEDDER, THOMAS; TUSCANO, JO) 4 September 2003 (2003-09-04)  page 18, paragraph 5 - page 19, paragraph 1 page 19, paragraph 3 - page 22, last paragraph -----	1-10,13, 15,20, 23-30, 33-49, 56-67
Y	WO 00/74718 A (IMMUNOMEDICS, INC; GOLDENBERG, DAVID, M; HANSEN, HANS, J) 14 December 2000 (2000-12-14)  page 16, line 19 - page 17, line 14 page 8, line 17 - page 10, line 17 abstract -----	1-10,13, 15,20, 23-30, 33-49, 56-67
A	WO 03/027135 A (THE GOVERNMENT OF THE UNITED STATES, AS REPRESENTED BY THE SECRETARY O) 3 April 2003 (2003-04-03)  abstract page 3, line 16 - page 4, line 7 page 31, line 20 - page 32, line 14 ----- -/--	1-10,13, 15,20, 23-30, 33-49, 56-67

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## INTERNATIONAL SEARCH REPORT

International application No

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Y	WO 03/075855 A (EOS BIOTECHNOLOGY, INC; BHASKAR, VINAY; DE LA CALLE, AGUSTIN; LAW, DEB) 18 September 2003 (2003-09-18)  page 27, last paragraph - page 28, paragraph 1 page 4, paragraph 3 page 3, paragraph 1 abstract	1-10, 13, 15, 20, 23-30, 33-49, 56-67
Y	US 5 416 064 A (CHARI ET AL) 16 May 1995 (1995-05-16)  column 12, line 27 - line 54	1-10, 13, 15, 20, 23-30, 33-49, 56-67
E	WO 2005/049075 A (GENENTECH, INC; CROWLEY, CRAIG; DESAUVAGE, FREDERIC, J; EATON, DAN, L;) 2 June 2005 (2005-06-02)  page 70, line 24 - line 35; examples 11, 12 page 117, line 26 - page 118, line 20 page 139, line 8 - page 140, line 19 page 142, line 6 - line 28	1-8, 10, 15, 20, 23-28, 30, 33-35, 39-44, 48, 64-66
E	WO 2005/101017 A (KADKHODAYAN, MIRYAM; MANN, EMILY; GENENTECH, INC) 27 October 2005 (2005-10-27) page 18, line 37 - page 25, line 39 page 31, line 19 - page 33, line 5 page 43, line 5 - page 44, line 8 page 46, line 1 - line 8 page 48, line 3 - line 15 page 51, line 33 - page 52, line 2	1-26, 30-43, 64-67

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## Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 35-55 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:  
1-10, 13, 15-67 (partially) and 11 (completely)
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Invention 1: claims 1, 3-8, 10, 15-64, 66 (partially) and 11 (completely)

Subject-matter related to an antibody-drug conjugate wherein the antibody binds to an ErbB receptor and wherein the linker has the structure of the first molecule mentioned in claim 3 (first molecule of the first line of molecules of claim 3 (-maleimide-X-CO-)) and wherein X is -CH<sub>2</sub>-cyclohexane-

---

Invention 2: claims 1, 3-7, 15-64 (partially)

Subject-matter related to an antibody-drug conjugate wherein the antibody binds to an ErbB receptor and wherein the linker has the structure of the first molecule mentioned in claim 3 (first molecule of the first line of molecules of claim 3 (-maleimide-X-CO-)) and wherein X is -(CH<sub>2</sub>)<sub>n</sub>-

---

Invention 3: claims 1, 3-7, 15-64 (partially)

Subject-matter related to an antibody-drug conjugate wherein the antibody binds to an ErbB receptor and wherein the linker has the structure of the first molecule mentioned in claim 3 (first molecule of the first line of molecules of claim 3 (-maleimide-X-CO-)) and wherein X is -(CH<sub>2</sub>-CH<sub>2</sub>-O)<sub>n</sub>-

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Invention 4: claims 1, 3-7, 15-64 (partially)

Subject-matter related to an antibody-drug conjugate wherein the antibody binds to an ErbB receptor and wherein the linker has the structure of the first molecule mentioned in claim 3 (first molecule of the first line of molecules of claim 3 (-maleimide-X-CO-)) and wherein X is -CH<sub>2</sub>-cyclohexane-CO-NR-(CH<sub>2</sub>)<sub>n</sub>- or -(CH<sub>2</sub>)<sub>n</sub>-CO-NR-(CH<sub>2</sub>)<sub>n</sub>-

---

Invention 5: claims 1, 3-7, 15-64 (partially)

Subject-matter related to an antibody-drug conjugate wherein the antibody binds to an ErbB receptor and wherein the linker has the structure of the first molecule mentioned in claim 3 (first molecule of the first line of molecules of claim 3 (-maleimide-X-CO-)) and wherein X is -benzene- or -benzene-(CH<sub>2</sub>)<sub>n</sub>-

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Invention 6: claims 1, 3-6, 9, 15-64 (partially) and 12 (completely)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Subject-mater related to an antibody-drug conjugate wherein the antibody binds to an ErbB receptor and wherein the linker as the structure of the second molecule mentionned in claim 3 (second molecule of the first line of molecules of claim 3 (-CH<sub>2</sub>-CO-Y-CO-))

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Invention 7: claims 1, 3-6, 13, 15-64, 67 (partially) and 14 (completely)

Subject-mater related to an antibody-drug conjugate wherein the antibody binds to an ErbB receptor and wherein the linker as the structure of the third molecule mentionned in claim 3 (first molecule of the second line of molecules of claim 3 (-maleimide-X-CH<sub>2</sub>-CH<sub>2</sub>-maleimide-))

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Invention 8: claims 1, 3-6, 15-64 (partially)

Subject-mater related to an antibody-drug conjugate wherein the antibody binds to an ErbB receptor and wherein the linker as the structure of the fourth molecule mentionned in claim 3 (second molecule of the second line of molecules of claim 3 (-CH<sub>2</sub>-CO-))

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Invention 9-43: claims 1-10, 13, 15, 20, 23-30, 33-67 (all partially)

Subject-mater related to an antibody-drug conjugate wherein the antibody binds to one of the molecules (1)-(16), (18)-(36) according to claim 1, each of the binding molecules defines one independant invention (the molecule (17) is not included since Her2 is an erbB receptor and therefore belongs to the inventions 1-8).

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Information on patent family members

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